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ABSTRACT

Title of Thesis: Sex Differences and the Effects of Stress on Subsequent Opioid Consumption in Adult Rats Following Adolescent Nicotine Exposure: A Psychopharmacologic Examination of the Gateway Hypothesis

Laura Cousino Klein, Doctor of Philosophy, 1997

Thesis directed by: Neil E. Grunberg, Ph.D.

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The present experiment examined effects of nicotine administration during adolescence on subsequent opioid consumption in male and female rats. Forty-one day old rats received saline (n = 40), 6 mg nicotine/kg/day (n = 40), or 12 mg nicotine/kg/day (n = 40) by osmotic minipump for 24 hours/day for 19 days. After a 7-day cessation period, consumption of fentanyl-HCl solution was evaluated for 4 weeks. Throughout the opioid consumption phase, rats received either 20 minutes of immobilization stress (n = 60) or no-stress (n = 60) prior to opioid availability. Body weight, food, and water consumption were evaluated throughout the experiment.

Nicotine exposure (6 mg nicotine/kg/day) during adolescence was related to increased, subsequent fentanyl self-administration in non-stressed male rats. Exposure to immobilization stress prior to opioid availability attenuated or reversed the effect of adolescent exposure to 6 mg nicotine/kg/day on fentanyl self-administration in adult male rats. These effects did not occur for female rats. Female rats consumed more fentanyl

than did male rats, regardless of nicotine pre-exposure, but male and female rats did not display differences in withdrawal following naloxone challenge. Opiate self-administration decreased food consumption for all animals. Nicotine history appeared to increase plasma corticosterone levels in non-stressed, male and female rats. Nicotine decreased body weight gains and food consumption among male and female rats and both of these effects were greater in female than in male rats. Nicotine cessation resulted in increases in body weight and food consumption and these effects were greater in females than in males. Stress increased plasma corticosterone in male and female rats and female rats had higher levels of plasma corticosterone. Stress decreased body weight and food consumption in male and female rats regardless of nicotine history.

The findings suggest that nicotine exposure during adolescence could increase opioid consumption in non-stressed males, but that other variables are likely to contribute to the progression from tobacco use to other drug use under conditions of stress and in females. The present experiment provides an animal model to help further investigate the psychopharmacologic effects of adolescent nicotine exposure on drug abuse and other appetitive behaviors in adulthood.

Sex Differences and the Effects of Stress on Subsequent Opioid Consumption in Adult Rats Following Adolescent Nicotine Exposure: A Psychopharmacologic Examination of the Gateway Hypothesis

by

Laura Cousino Klein

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Department of Medical and Clinical Psychology
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of the requirements for the degree of
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I find irony in the juxtaposition of this one page, meant to hold all of the appreciations, meaningfulness, and memories of those who supported me in this process, against the several other pages that create the bound thesis. There simply is no comparison. This dissertation represents a culmination of achievements, failures, and countless lessons -- the easy and the not-so-easy ones. It is a process that could not have happened without the commitment and support of some very special individuals. Mom and Dad, thank you for believing in me and for your never-ending love, encouragement, and support; Kelly, thank you for your gift of friendship and the honor of being your colleague; and Neil, thank you for the unconditional gift of being my mentor, a continuously changing and profoundly complex role.

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INTRODUCTION

Cigarette smoking is the single most preventable cause of death and illness in the United States and it is responsible for an estimated 400,000 deaths annually (Grunberg, Brown, & Klein, 1997; USPHS, 1992). It is estimated that roughly half a billion people alive in the world today eventually will die from tobacco use (Peto & Lopez, 1990) and that smoking will contribute to an annual toll of 10 million deaths by the year 2020 (Grunberg et al., 1997). Yet despite the health risks involved, 25% of Americans continue to smoke (CDC, 1991; USDHHS, 1994) and it is estimated that the health care costs and lost productivity as a result of tobacco use in the U.S. alone amount to about $65 billion a year (US Congress Office of Technology Assessment, 1985). The tobacco industry has to recruit 3,000 to 5,000 new smokers daily to help compensate for the profits lost to smokers who die and the 1.5 million Americans that decide to quit on their own each year (USDHHS, 1994). Nearly 90% of all smokers begin smoking during adolescence and current reports estimate that 3,000 adolescents start smoking every day (Lynch & Bonnie, 1994; USDHHS, 1994). There has been little change in smoking prevalence among adolescents since 1991 and as many or more adolescent girls now smoke as do adolescent boys (Glynn, 1993; Nelson et al., 1995; USDHHS, 1994). In fact, over 25% of 17- and 18-year olds in the United States currently smoke (USDHHS, 1994).

Cigarette smoking and tobacco use also have been reported to precede the use of illicit drugs, including opioids, and have been hypothesized to be a "gateway" to the use of these other drugs (Kandel, 1975). It has been suggested that adolescents who smoke cigarettes are more likely to subsequently use and abuse illicit drugs of dependence. Other

than epidemiologic reports, there have been no causal, empirical analyses of exposure to tobacco on subsequent illicit drug self-administration. Whether these behaviors are simply correlational or whether use of tobacco causally influences subsequent use of other drugs has not been determined. Examination of a causal hypothesis is important to determine whether prevention of tobacco use would influence use of other drugs and also to determine how exposure to tobacco (or key constituents of tobacco) affects self-administration of other drugs.

There are several variables that could contribute to the relationship between cigarette smoking during adolescence and subsequent drug use, including social, cultural, and biological factors. Animal models can be used to examine biologic mechanisms related to drug abuse, including exposure to the primary addictive ingredient in cigarettes, nicotine. Using a nicotine administration paradigm in rats that has accurately reflected behaviors of human smokers, the present experiment was designed to examine the gateway hypothesis in a causal model. Specifically, the effects of nicotine exposure during adolescence on opioid consumption in adult male and female rats exposed to stress or not exposed to stress was examined. Background and support for selection of these specific drugs, the inclusion of two sexes, and the inclusion of stress as a potential mediator of drug self-administration are presented before the experimental protocol. First, the gateway hypothesis is presented. Next, the role of nicotine and tobacco use and the rationale for manipulating nicotine *per se* is presented. This section includes a discussion of the potential sex differences in nicotine's effects and psychopharmacologic mechanisms of action for the gateway hypothesis. Then, a discussion of the class of drugs known as

opioids is provided, including a rationale for the selection of fentanyl (an opioid) in the present experiment. The next section discusses stress and the relationship between stress and opioid self-administration. An overview of the experiment is provided along with specific hypotheses and a detailed methods section. Finally, results of the experiment are presented, followed by a discussion of the results and potential significance for understanding the gateway hypothesis.

Gateway Hypothesis

Clinical and epidemiological reports indicate that cigarette smoking and/or alcohol consumption during adolescence play a prominent role in the subsequent use of illicit drugs, such as cocaine and heroin (Blaze-Temple & Lo, 1992; Kandel, 1975; Kandel, Marguilies, & Davies, 1978; Kandel & Yamaguchi, 1985; Kandel, Yamaguchi, & Chen, 1992; Newcomb & Bentler, 1986). Further, retrospective reports by Kandel and colleagues (Kandel & Yamaguchi, 1993; Kandel et al., 1992) indicate that progression to the abuse of illicit drugs may be different for men and women. Specifically, abuse of illicit drugs by young men was mostly correlated with alcohol use during adolescence, whereas cigarette smoking and/or alcohol consumption was a sufficient initial experience for women to progress to the abuse of illicit drugs.

These data have been collected over the past 20 years, primarily on inner-city youth from New York City. Although these reports indicate that there is a positive link between cigarette smoking and subsequent abuse of illicit substances, these studies only provide correlational, self-report information that does not allow for causality to be determined. Specifically, it is difficult to determine whether adolescent biological, social,

or cultural factors, either individually or separately, play an important role to determine illicit drug use in adulthood. Interestingly, the results do suggest that individual difference variables, gender and age in particular, play an important role in the progression from cigarette smoking to drug use and/or abuse.

Unfortunately, several questions still remain regarding the relationship between smoking a cigarette and using illicit drugs. It could be that: (1) cigarette smoking and other drug use simply co-occur without causality; (2) cigarette smoking precedes other drug use because tobacco products are readily available to adolescents, whereas illicit drugs become available only after adolescence; (3) progression from cigarette smoking to drug use is a consequence of boredom; (4) there are cultural and social influences associated with smoking and consequent drug use that become reinforcing for some adolescents; or (5) cigarette smoking has some biological effect that increases the likelihood of subsequent drug use. It is this last possible explanation that is the focus of the present research.

A psychopharmacologic examination of the gateway hypothesis. There are several scientific advances that allow for a psychopharmacologic examination of the gateway hypothesis. The 1988 Surgeon General's Report (USDHHS, 1988) clearly reviewed the evidence that nicotine is addictive and that smokers smoke to self-administer nicotine. Nicotine also is the primary active pharmacologic agent in tobacco products and it was hypothesized in the present experiment that any direct effect of cigarette smoking and tobacco exposure on subsequent drug consumption is likely a result of the effects of nicotine. Nicotine's effects in animals (e.g., body weight, food consumption, attention,

physical activity) have been replicated in human smokers and a rat model of nicotine administration is available that has produced findings that are similar to human smokers (Grunberg, 1982; USDHHS, 1988). In addition, paradigms are available to examine drug self-administration in adult rats that have helped to investigate the causal relationship between stress and drug use (Alexander, Coambs, & Hadaway, 1978; Klein, Popke, & Grunberg, 1997; Shaham, Alvares, Nespor, & Grunberg, 1992; Shaham, Klein, Alvares, & Grunberg, 1993). To date, these two areas of drug research have not been brought together and, therefore, no animal model has been developed to investigate the causal relationship between nicotine exposure during adolescence and subsequent self-administration of an opiate by males and females. The primary purpose of the present experiment was to develop this psychopharmacologic animal model. Specifically, nicotine was administered to adolescent male and female rats and then these animals were given an opportunity to self-administer opioids when they became adults. It is noteworthy that animals were not given nicotine when they were adults. That is, nicotine administration occurred during adolescence and was not concurrent with availability of opioids. Although teens do not quit smoking before they go on to use other drugs, the gateway hypothesis is based on the premise that smoking (i.e., nicotine administration) during adolescence *precedes* the use of illegal drugs as an adult. It is possible that smoking potentiates the reinforcing value of other drugs and that concurrent nicotine administration is a necessary condition for abusing other drugs. However, in order to determine the possible causal influence of nicotine exposure during adolescence on subsequent opioid consumption without the influence of nicotine in the system, the present experiment

limited nicotine exposure to adolescence.

Gateway hypothesis and stress. An additional purpose of this experiment was to investigate the effects of stress on opioid consumption and whether or not this relationship was influenced by prior nicotine exposure. Although no investigation of the gateway hypothesis in humans has directly evaluated stress as it pertains to illicit drug consumption, this experiment investigated the influence of stress on opioid consumption following a history of nicotine exposure. It has been suggested that there are several commonalities between stress and substance use (Baum & Grunberg, 1985) and reports with humans and animals suggest that there is a positive relationship between stress and substance abuse (Kosten, Rounsaville, & Kleber, 1986; O'Doherty, 1991; Shaham et al., 1992; Shaham et al., 1993; Shaham & Stewart, 1994; Shaham & Stewart, 1995; Shiffman & Wills, 1985). In addition, many smokers report that they smoke more when they are stressed. Because the effects of stress on the gateway hypothesis have not been investigated, no *a priori* predictions regarding nicotine history on the effects of stress on opioid self-administration were available. However, the introduction section of this dissertation ends with a discussion of the relationship between stress and substance abuse, and hypotheses are developed regarding the effects of stress on opioid consumption.

Nicotine and Smoking

Nicotine is a powerful, toxic pharmacologic agent that, when introduced into the body, acts in the brain and the periphery. Nicotine exists in the leaves of the *Nicotiana tabacum* (i.e, tobacco) plant. Humans self-administer nicotine by smoking processed tobacco leaves in the form of various tobacco products (e.g., cigarettes, pipe tobacco,

cigars), by other oral means (e.g., chewing tobacco, snuff), or by the use of nicotine-containing products (e.g., nicotine polacrilex gum, nicotine patch, nicotine nasal spray) (USDHHS, 1988). In addition to nicotine, tobacco smoke contains over 4,000 chemicals. Many of these chemicals are biologically active and contribute to the health hazards of smoking, but it is nicotine that is the primary pharmacologic agent of addiction and it is nicotine that is considered to be the most behaviorally-relevant pharmacologic agent in tobacco products (Grunberg et al., 1997; USDHHS, 1988). In fact, it is now well-established that:

> (1) Cigarettes and other forms of tobacco are addicting;
>
> (2) nicotine is the drug in tobacco that causes addiction; and
>
> (3) the pharmacologic and behavioral processes that determine tobacco addiction are similar to those processes that determine addiction to other drugs such as heroin and cocaine (p. 9, USDHHS, 1988).

Pharmacokinetics of nicotine. Nicotine is composed of a pyridine and pyrrolidine ring (see Figure 1) and its absorption rate across lipid membranes in the body varies as a function of pH. Nicotine (162.23 molecular weight) is a weak base with a pKa (an index of ionic dissociation) of 8.0 (aqueous solution at 25° C) (USDHHS, 1988). In its nonionized state, nicotine readily crosses lipid membranes, including the blood-brain barrier. Nicotine is quickly absorbed in the lungs through tobacco smoke or through the mouth or nose from smokeless tobacco. Once nicotine enters the pulmonary circulation, it is rapidly delivered to the brain and it exerts its effects in the central nervous system (e.g.,

hippocampus, thalamus, nucleus accumbens). In addition, nicotine is distributed throughout the body by the circulatory system and acts at peripheral sites (Benowitz, 1987; USDHHS, 1988).

Pharmacodynamics of nicotine. Nicotine is a potent psychoactive drug that has a cascade of central and peripheral effects when it is administered in concentrations that are found in tobacco (Kumar & Lader, 1981; Balfour, 1984; USDHHS, 1988). Nicotine acts at nicotinic cholinergic receptors (nAChR) in the periphery at neuromuscular junctions and endplates (USDHHS, 1988). The peripheral effects of nicotine begin with stimulation of peripheral cholinergic ganglia of the autonomic nervous system (ANS) and results in general sympathetic nervous system (SNS) arousal including: (1) increased heart rate and blood pressure; (2) vasoconstriction in the distal extremities; (3) skeletal muscle relaxation and constriction; (4) respiratory enhancement or failure (at toxic doses); and (5) increases in secretions of the gastrointestinal tract and decreased gastric motility (USDHHS, 1988). In the brain and the central nervous system (CNS), nicotine acts at several different receptor types. The principle central nAChR is the $\alpha 4_3 \beta 2_2$ *Torpedo* receptor but there also are other relevant nAChRs, including the $\alpha 3 \beta 2$ and $\alpha 7$ receptor sites. These nAChRs are distributed in several brain regions, including the limbic region, the thalamus, and regions related to nicotine's rewarding effects (i.e., ventral tegmental area, nucleus accumbens; Lindstrom et al., 1995; USDHHS, 1988).

Nicotine administration releases catecholamines, corticosteroids, neuropeptides, and pituitary hormones, attenuates serotonin turnover, and typically results in electrocortical activation (Grunberg et al., 1997; USDHHS, 1988). Nicotine

administration also stimulates dopamine release from the ventral tegmental area (Nisell, Nomikos, & Svensson, 1994; Yoshida et al., 1993), the striatum (Blaha & Winn, 1993), and the nucleus accumbens (Yoshida et al., 1993; Nisell et al., 1994). All of these effects are thought to contribute to the reinforcing actions of nicotine self-administration by tobacco use.

In addition to nicotine's dopaminergic and other biochemical actions that are likely to contribute to its reinforcing effects, nicotine is a positive reinforcer because it suppresses appetite for specific foods, controls body weight, and enhances attention. Nicotine acts as a negative reinforcer by attenuating unpleasant withdrawal effects of smoking cessation, including irritability and loss of concentration (Grunberg et al., 1997; USDHHS, 1988; West & Grunberg, 1991). Principles of learning and conditioning also act so that environmental, social, and psychological cues associated with tobacco smoking (and nicotine self-administration) can elicit similar biological responses (Grunberg et al., 1997; USDHHS, 1979; USDHHS, 1988). Although effects of nicotine are robust, individual difference variables such as race, gender, and age can alter effects of nicotine (e.g., Gritz, 1986; Grunberg, Winders, & Wewers, 1991; Grunberg et al., 1997; USDHHS, 1988).

Smoking prevalence by socioeconomic status, age, and gender. In the United States, lower socioeconomic status (SES) groups smoke at high prevalence rates and the marked decreases in smoking prevalence only have occurred among upper SES groups (Grunberg et al., 1997). Among American youth, the news also is alarming. Despite overall decreases in smoking prevalence rates among the general U.S. population,

smoking is not decreasing among minors and about 3,000 adolescents start to smoke every day (USDHHS, 1994). In 1994, at least 3.1 million adolescents smoked and used tobacco (USDHHS, 1994). The number of adolescent smokers, however, is not equally distributed among subgroups. Specifically, smoking prevalence among African-American youth is steadily decreasing, whereas smoking among white adolescents is higher and is on the rise (Grunberg et al., 1997). With this high number of adolescents smoking and great numbers of youths starting every day, it is particularly important to evaluate the gateway hypothesis to determine whether this tobacco use among adolescents is a harbinger of an enormous rise in drug use and abuse.

With regard to cigarette smoking and gender, the statistics have changed dramatically over the past 40 years. Today, about 25% of American adults smoke cigarettes with comparable prevalence among men and women. Roughly 1 out of 4 men and 1 out of 4 women smoke today (Grunberg et al., 1997; Grunberg & Klein, in press; USDHHS, 1989). These statistics are noteworthy when one considers the fact that fewer women smoked over 40 years ago, whereas more than 50% of men smoked 40 years ago (OSH, 1991; USDHHS, 1989). The most striking statistic, however, is that as many or more adolescent girls smoke today as do adolescent boys (USDHHS, 1994). This statistic suggests that, among American adults, more women than men may smoke by the year 2000 (Grunberg & Klein, in press; USDHHS, 1994). If the gateway hypothesis is confirmed, then the future prevalence of illicit drug use by gender may change from current statistics that indicate more men than women using illicit drugs (Lex, 1991).

There are many reasons for the gender shift in smoking trends, and cigarette use is

a result of different factors including: availability of tobacco products, perceived benefits of smoking, perceptions of the risks associated with smoking, attitudes towards smoking, and the psychopharmacologic effects of cigarettes. A better understanding of the pharmacologic, psychologic, and biologic mechanisms that underlie tobacco use in males and females could help prevent these marked changes in smoking initiation and subsequent persistence of smoking behavior and other drug use.

There is a sad example that illustrates how a lack of understanding of these mechanisms can have opposite consequences from those intended. In the 1970s, public health proponents strongly encouraged a decrease in the nicotine and tar yields of cigarettes in an attempt to protect people from the dangers of smoking. This position was based on the logic that less nicotine and tar content should decrease the amount of exposure to the toxic effects of a cigarette. Unfortunately, it was not considered that smokers are addicted to cigarettes and that they smoke to self-administer nicotine. Consequently, reduction of nicotine content results in smokers adjusting their smoking topography (i.e., depth of inhalation on each puff, number of puffs taken, number of cigarettes smoked) in order to receive the desired amount of nicotine levels (Kozlowski, Rickert, Robinson, & Grunberg, 1980). This compensatory change in smoking behavior actually delivers more of the toxic effects to the smokers than if they had smoked a higher nicotine and tar yield cigarette. In addition, women seem to be more sensitive than men to nicotine (Bättig, Buzzi, & Nil, 1982; Silverstein, Feld, & Kozlowski, 1980). Therefore, women actually may smoke more when nicotine yields are low and they may smoke less when the yields are high (Kozlowski et al., 1980; Grunberg & Klein, in press). Because

the continued pattern of smoking is dependent on initial, positive experiences with smoking a cigarette, it is possible that this gender difference in sensitivity may have contributed to the continued increase in smoking initiation among females with the increase in availability of low nicotine cigarettes. That is, females may have a more pleasant initial experience (or less unpleasant initial experience) with smoking a cigarette if they select a low nicotine cigarette, thereby increasing the likelihood that they will smoke again.

Sex differences in sensitivity to nicotine. Interestingly, investigations with rats have revealed sex differences with nicotine on body weight, food and water consumption, behavioral effects, and central neurochemistry that are consistent with human studies (Bättig, 1981; Grunberg, Bowen, & Winders, 1986; Grunberg, Winders, & Popp, 1987; Levin, Morgan, Galvez, & Ellison, 1987; Rosecrans, 1971; Rosecrans, 1972). Specifically, females (rats and humans) appear to be more sensitive to effects of nicotine (Bättig, 1981; Silverstein et al., 1980). There are two possible ways that females may be pharmacologically more sensitive to the effects of nicotine. First, the female dose-response curve to nicotine may be shifted to the left of the male dose-response curve. In this case, females reach their dose-response peak at lower nicotine dosages than do males. For example, females need a lower dosage of nicotine in order to place them at a point (i.e., ascending or descending limb) on the dose-response curve that is similar to males. Second, it could be that the female dose-response curve has a higher peak than does the male dose-reponse curve. In this second case, females display heightened responses to a similar dose of nicotine compared to males. There are no data to suggest which dose-

response curve underlies this female sensitivity to nicotine. However, human data suggest that nicotine may be less reinforcing for women compared with men (Perkins, 1996). This gender difference in sensitivity to nicotine also may apply to effects of nicotine administration on later drug self-administration. That is, there may be gender differences in the gateway hypothesis. The fact that these and other effects of nicotine have been revealed in rat studies and replicated with human cigarette smokers indicates that animal paradigms can be used to provide meaningful information regarding behavioral and biological effects of nicotine.

An animal model to investigate effects of nicotine. Animal models provide an opportunity to investigate underlying mechanisms that simply cannot be examined in humans. For example, it would be unethical to expose young boys and girls to various nicotine yield cigarettes and examine whether or not they continue to smoke, use other drugs, and develop different smoking-related diseases. Several animal models have been used to investigate the effects of nicotine on neurobiological, physiological, and behavioral effects of nicotine. One paradigm, developed by Grunberg (1982) to investigate the effects of nicotine on body weight, has provided results on appetitive behaviors, activity, and attention that have been replicated in humans (e.g., Gritz, Klesges, & Meyers, 1989; Grunberg, 1992; Grunberg, Bowen, Maycock, & Nespor, 1985; Grunberg, Bowen, & Morse, 1984; Grunberg, Popp, Bowen, Nespor, Winders, & Eury, 1988; Grunberg et al., 1987; Klesges & Klesges, 1988; Klesges, Meyers, Klesges, & LaVasque, 1989; Klesges, Meyers, Winders, & French, 1989; Winders & Grunberg, 1989).

This animal paradigm administers nicotine subcutaneously through Alzet osmotic

minipumps (Alza Corporation, California). These devices slowly (0.5 μl/hour) release their contents (e.g., saline, nicotine solutions) for 1-3 weeks depending on the model of the minipump. This slow content delivery allows administration of a high dosage of nicotine, similar to the high amounts that a long-term, habitual human smoker would self-administer. The osmotic minipump's continuous administration establishes long-term, stable levels of nicotine in the animal rather than the acute bolus that is self-administered by smokers.

Some researchers suggest that repeated bolus administration of nicotine (i.e., peaks and troughs of nicotine levels) are necessary to establish nicotine addiction, whereas` others investigators have indicated that steady plasma levels of nicotine, similar to those established by the minipump preparation, are sufficient for nicotine addiction (USDHHS, 1988). Further support for the effectiveness of continuous nicotine administration stems from evidence that over-the-counter preparations that slowly release nicotine, like the transdermal nicotine patch, are effective in offsetting nicotine withdrawal in some smokers who have quit smoking. Another difference between the minipump preparation and the human smoker is that smokers voluntarily self-administer nicotine, whereas animals receive nicotine parenterally. However, despite these differences in pattern of drug delivery between the minipump and nicotine self-administration by smokers, the minipump paradigm was used in adolescent rats to evaluate the gateway hypothesis for several reasons: (1) the minipump model of nicotine administration yields results in rats that have been replicated in human laboratory and clinical studies (Gritz et al., 1989; Grunberg, 1992; Grunberg et al, 1985; Grunberg et al, 1984; Grunberg et al.,

1988; Grunberg et al., 1987; Klesges & Klesges, 1988; Klesges et al., 1989; Klesges et al., 1989); (2) the minipump preparation is easier to use than models of nicotine self-administration or repeated nicotine injections, this paradigm minimizes differences in nicotine dosages within drug treatment groups, and it decreases extraneous factors associated with frequent drug injections; (3) more animals can be included in the experiment because of the simplicity of the paradigm; (4) the minipump paradigm has been shown to deliver nicotine dosages that produce plasma nicotine levels comparable to human smokers; and (5) it was important to determine whether or not nicotine exposure *per se* was an important pharmacologic variable in the gateway hypothesis. The dosages of nicotine that were used in this experiment, the correlation of nicotine dosages in rats with dosages found in human smokers, and the methods for nicotine administration are based on previous reports and are discussed in the methods section of this dissertation.

Possible biologic links between nicotine exposure and subsequent drug use. The opponent process theory (Solomon & Corbitt, 1974) may help explain how nicotine exposure increases opioid consumption. Specifically, increased opioid consumption may occur when repeated exposure to nicotine results in: (1) a decrease in the rewarding effects of nicotine (e.g., tolerance, habituation; response A), and (2) an increase in the negative effects of nicotine administration (e.g., withdrawal; response B). Therefore, opioid consumption could be used to offset the negative effects of nicotine (i.e., response B).

Subsequent opioid use also may occur as a result of cross-dependence, cross-tolerance, or cross-sensitization to the rewarding actions of nicotine. Specifically,

research suggests that dopamine plays an important role in reward processes and that both nicotine and opioid administration increase dopamine in the brain areas (e.g., ventral tegmental area, nucleus accumbens) believed to be important in drug-reward processes (Bozarth & Wise, 1986; Cox & Werling, 1990; Di Chiara & Imperato, 1988; Jaffe, 1990; Lindstrom et al., 1995; Nisell et al., 1994; Yoshida et al., 1993).

Opioids

Opium is a crude substance derived from the seed pod of the opium poppy *Papaver somniferum*. The pod is sliced after the flower petals have dropped and a white latex oozes from the incision. Eventually, the latex hardens and turns brown and this sticky, gum-like substance is called opium. It is believed that the plant has been used for over 6,000 years and its use in Greek, Roman, and Egyptian cultures has been documented. In 1803, Sertürner, a German pharmacist, isolated a pure alkaloid substance from opium that was pharmacologically active. He named the compound "morphine" after the Greek god of dreams, Morpheus (Jaffe & Martin, 1990; Way & Way, 1992).

It is now known that the principal alkaloid in opium is morphine. Morphine has been used clinically for over a hundred years because of its ability to provide relief of severe pain, control diarrhea, treat cough and insomnia, and diminish anxiety. Opioid analgesics are a group of drugs that are morphine-like or opium-like in their pharmacologic properties. This drug class includes both natural (e.g., morphine) and semisynthetic (e.g., heroin) alkaloid derivatives from opium. Also included in this class of drugs are synthetic (e.g., methadone and fentanyl) drugs that have actions which mimic the effects of morphine. The term *opioid* refers to all substances, natural, semi-synthetic, and

synthetic, that exert morphine-like actions, whereas the term *opiate* is used to describe drugs that are derived from the opium alkaloids (e.g., morphine). Endogenous opioid peptides (i.e., opioipeptins) such as endorphins, enkephalins, and dynorphins also belong to the opioid class (Jaffe & Martin, 1990).

Opioid drugs are used clinically to alleviate pain, cough suppression, and provide symptomatic relief of diarrhea (Cox, 1990; Jaffe & Martin, 1990). Other actions of opioids include respiratory depression, euphoria, sedation, immunosuppression, mood changes, decreased aggression, drowsiness, and endocrinological changes (Jaffe, 1985; Jaffe & Martin, 1990; Way & Way, 1992). Tolerance to these effects occurs with repeated use of morphine and morphine-like drugs. Opioids are considered the gold standard of addictive drugs and there are approximately 600,000 opioid addicts in the United States, as well as 2,000,000 opioid abusers. Fentanyl, a potent opioid agonist, was used in the present experiment.

Fentanyl. Fentanyl is a strong, synthetic opioid agonist compound whose basic chemical structure is a phenylpiperidine (see Figure 2). Fentanyl was first synthesized in the 1960s and it is the parent compound in the fentanyl subgroup of the phenylpiperidines (e.g., alfentanil, sufentanil). The fentanyls were introduced into the United States in the 1970s and are commonly used in the relief pain and as an anesthetic (Henderson, 1990).

Opioids, like morphine and fentanyl, act at receptors that are located in the brain, spinal cord, and gastrointestinal tract. There are several receptor types that have been characterized, including mu (μ), delta (δ), kappa (κ), lambda (λ), and epsilon (ϵ) receptors (Cox & Werling, 1991). Studies suggest that the μ receptor mediates analgesia,

respiration, and thermoregulation. More recently, it has been suggested that the mu receptor subtype, μ_2, plays a role in reward-mediated behaviors (like drug-taking) via dopaminergic pathways (Suzuki, Funada, Narita, Misawa, & Nagase, 1993).

Fentanyl is primarily a μ-opioid receptor agonist that is 80-100 times more potent than morphine as an analgesic. Fentanyl is a small molecule with a pKa of 7.7 and it has a short half-life (approximately 3.7 hours). Fentanyl has a high lipid solubility and, therefore, it crosses the blood-brain barrier rapidly (within a few minutes) regardless of the route of entry into the body. Once in the system, fentanyl is quickly absorbed in the gastrointestinal tract and both its analgesic effects and euphoric effects are antagonized by opioid receptor antagonists such as naloxone (Jaffe & Martin, 1990). Chronic administration of fentanyl results in drug tolerance. Specifically, a state of decreased responsiveness to the pharmacologic effect of the drug occurs after prior exposure to the drug (Cox, 1990).

The fentanyls were introduced in California in 1979 as illicit drugs (e.g., "China White" or "synthetic heroin"). These drugs are attributed with over 100 deaths in the 1980s and fentanyl is abused among some health care professionals. Henderson (1988) reported that fentanyl was the primary substance abused by anesthesiologists and several clinicians report that the first sign of fentanyl addiction is death by drug overdose (David Hester, personal communication, 1996). The addiction and abuse liability of fentanyl is high (Way & Way, 1992) and cessation of the drug in dependent humans results in severe withdrawal symptoms that last from 7 to 10 days. This withdrawal syndrome is manifested by sympathetic nervous system hyperactivity such as: restlessness, drug

craving, yawning, runny nose, chills, fever, loss of appetite, insomnia, hypertension, anxiety, and dysphoria (Cox, 1990; Jaffe, 1985). Opioid withdrawal can be precipitated either by cessation of drug-taking or injection of an opioid antagonist (i.e., naloxone or naltrexone) while the opioid agonist is in humans and animals (Jaffe & Martin, 1990; Klein, Popke, & Grunberg, 1997; Linseman, 1977; Shaham, Alvares, Nespor, & Grunberg, 1992; Shaham, Klein, Alvares, & Grunberg, 1993).

Because of its pharmacokinetic and pharmacodynamic properties, fentanyl is an excellent opioid compound to use in animal self-administration paradigms. Specifically, it quickly gets into the system, has a short half-life, and therefore, it is gone by the time subjects are exposed to experimental conditions on the subsequent day. When dissolved in water, fentanyl hydrochloride (HCl) is less bitter-tasting than morphine and it is readily self-administered orally by adult male and female rats (Klein et al., 1997; Shaham et al., 1992; Shaham et al., 1993).

Stress

The earliest systematic investigation of stress was conducted by Cannon in the late 1920s (Cannon, 1935). Cannon defined stress as a profile of emotional and physiological responses to danger and suggested that stress results in a disruption of homeostasis. For Cannon, the sympathetic nervous system and the adrenal medulla, in particular, were critical in the body's response to stress that he labeled the "fight-or-flight" response. In addition, stress could result in various medical problems. It is now known that consequences of exposure to stress can include pathophysiological responses (e.g., cardiovascular diseases, ulcers) to psychological or physical stress or behavioral responses

(e.g., drug-taking, changes in eating behavior) to stress in males and females (Glass & Singer, 1972; Gottdiener et al., 1994; Grunberg & Straub, 1992; Howell & Krantz, 1994; Klein et al., 1997; Krantz et al., 1993; Shaham et al., 1992; Shaham, 1993).

Selye (1955, 1956) conceptualized stress based on his work with different physical stressors, including cold, heat, and exercise. Selye emphasized the role of corticosteroids and the resulting pathophysiological responses. He concluded that any and all stressors elicit a "General Adaptation Syndrome" (GAS), characterized by adrenal enlargement, thymus gland shrinkage, and gastrointestinal ulcers (Selye, 1955, 1956). The GAS consists of three sequential stages of response, namely: an initial alarm reaction; a stage of resistance; and exhaustion if the stressor is prolonged. The initial alarm reaction is when the organism is first exposed to the stressor and prepares to resist it. Adrenal activity increases during this initial stage. During the stage of resistance the organism repeatedly attempts to deal with the stressor. If adaptation to the stressor is not achieved, then the organism's body enters a state of exhaustion in which there is a depletion of the body's adaptive reserves.

Mason (1974, 1975a, 1975b) challenged the idea that there is a non-specific response to all stressors and that the stress response is unidimensional. Mason reported that different stressors result in different endocrinological profiles. In addition, Mason (1975b) suggested that psychological factors, such as predictability, controllability, and perceived control, may alter the stress response.

Lazarus (1966) and Lazarus and Folkman (1990) emphasized that perception and appraisal of the stressor are critical to elicit the stress response. According to these

psychologists, unless the stressor is perceived as stressful, there will not be a stress response. Based on initial appraisal, a stressor can elicit different behavioral and psychological coping processes.

Modern concepts of stress hold that stressors can be psychological, physical, or environmental events that can threaten the organism's safety (Baum, Singer, & Baum, 1981). Stress is considered to be a process that includes three important elements: stressors, stress responses, and factors that may mediate the effects of stress on the organism (Baum, 1990; Baum, Grunberg, & Singer, 1982; Cohen et al., 1982; Grunberg & Singer, 1990). Stressors are defined as events (perceived or real) that disrupt the homeostasis of an organism. The disruption of this homeostasis is called the stress response. The stress response can be manifested on several levels: (1) physiological, such as increased catecholamine or corticosteroid secretions and increased blood pressure and heart rate; (2) psychological, such as depression or anxiety; and (3) behavioral, such as decreased performance on cognitive tasks, decreased persistence on frustrating tasks, and decreased attention (Baum, 1990; Baum et al., 1987; Cohen et al., 1986). Factors that may mediate the effects of stress on the organism range from individual factors such as personality traits, coping mechanisms, cognitive appraisal, and genetic predispositions, to environmental and social factors such as social support and predictability (Baum et al., 1982; Baum et al., 1987; Glass & Singer, 1972; Singer & Davidson, 1990). Because the stress response occurs on several levels, investigators have argued that the best approach for evaluation of stress responses is assessment of stress responses on many levels (Baum et al., 1982; Grunberg & Singer, 1990; Baum & Grunberg, 1995). For example, self-

reports of mood and stress, performance, behaviors (e.g., smoking, eating, drug-taking), physiological responses (e.g., blood pressure, heart rate), and biochemical responses (e.g., catecholamines, endogenous opioid peptides) can be used together to provide an overall picture of stress responding both in the natural setting and in the laboratory, in humans and animals. Underlying neurobiologic mechanisms play a role in stress responses and animal models are a valuable tool to help examine the relationships between stress, behavioral, biochemical, and biological responses.

Animal investigations of stress and opioid consumption. Clinical reports and observations suggest that there is a positive relationship between stress and drug use. These reports also suggest that stress might play an important role in drug relapse (Kosten, Rounsaville, & Kleber, 1986; O'Doherty, 1991; Shiffman & Wills, 1985; Whitehead, 1974). Unfortunately, these studies are limited by small sample sizes, insufficient control groups, and inadequate appraisal of stress responses (i.e., psychological, physiological, and behavioral assessment). In addition, epidemiological studies provide correlational information that does not allow for causal explanations to be addressed. Therefore, a causal relationship between stress and substance abuse cannot be determined from these studies (Hall, Havassy, & Wasserman, 1990; O'Doherty & Davies, 1987). A few investigators have considered mechanisms that might mediate the stress-substance abuse relationship in humans (Grunberg & Baum, 1985; Hall et al., 1990; O'Doherty & Davies, 1987). In addition, there are sex differences in the use and abuse of licit and illicit drugs (Grunberg et al., 1991; Lex, 1991).

Animal paradigms of drug self-administration are useful because naive subjects can

be given access to addictive drugs and the effects of stress on drug consumption can be evaluated directly. In the past, opioids were the gold standard to evaluate drug addiction. Dib and colleagues (Dib & Duclaux, 1982; Dib, 1985) were the first investigators to conduct investigations on physical stress and opioid consumption in rats. These investigators reported an increase in morphine self-administration by male rats during a footshock stressor. Because drug consumption was evaluated during footshock exposure and because morphine has analgesic effects, it is possible that the observed increase in drug consumption by the subjects was to decrease discomfort of the stressor, rather than to experience the reinforcing effects of the morphine.

In order to minimize the confounding effects of painful stress on opioid consumption, Grunberg and colleagues (Shaham et al., 1992; Shaham et al., 1993; Klein et al., 1993; Klein et al., 1997) developed additional animal paradigms to investigate the effects of stress on opioid consumption in adult rats. Specifically, a series of experiments were designed to examine oral opioid (morphine and fentanyl) consumption following stressor exposure (immobilization, unpredictable footshock, predictable footshock). It was hypothesized that assessment of drug consumption following cessation of the stressors would provide a better model of drug-taking behavior for the reinforcing effects of the opioids that was not related to the analgesic property of the drugs.

Shaham et al. (1992) reported that immobilization (IM) stress, prior to drug availability, increased home cage oral consumption of both morphine and fentanyl over no-stress control conditions in male rats. The next experiment extended this initial study by examining the effects of a different stressor (footshock) on opioid (fentanyl) consumption

in an operant paradigm (Shaham et al., 1993). The results suggest that male rats "work" harder for an opioid reinforcer following stress than they do following no-stress. In addition, responses for an equally bitter solution (quinine) or water following stress extinguished when these solutions were substituted for the opioid solution. These results suggest that the observed increase in responding for the opioid solution following stress was a result of the effects of the drug rather than a non-specific increase in activity (e.g., lever responding behavior), thirst (responding for water), or change in taste sensitivity (responding for quinine solution).

In order to examine whether or not this paradigm could be used for females, the next study examined the effects of footshock on fentanyl consumption in female rats (Klein, Shaham, Alvares, & Grunberg, 1993). This study found that female rats consumed more fentanyl following stress than they did following no-stress conditions.

The next study in this series was designed to replicate these previous findings and to examine whether or not a psychological variable (predictability) could mediate drug consumption in rats (Klein et al., 1997). Specifically, adult male and female rats were exposed to either predictable or unpredictable footshock and subsequent fentanyl consumption was measured. Results suggest that, regardless of stressor condition, females self-administered significantly more fentanyl than did males. This main effect for sex began on the third day of drug exposure and continued throughout the experiment. This sex difference replicated results from an earlier experiment using adult male and female rats in different environmental conditions (Alexander, Beyerstein, Hadaway, & Coambs, 1981; Alexander, Coambs, & Hadaway, 1978; Bozarth, Murray, & Wise, 1989;

Marks-Kaufman & Lewis, 1984). Surprisingly, following naloxone challenge (i.e., opioid antagonism), male rats exhibited significantly more withdrawal symptoms following injection than did females, despite lower amounts of fentanyl consumption. In addition, animals exposed to predictable footshock stress self-administered more fentanyl than did animals exposed to unpredictable footshock. Following a drug washout period and extinction of responding for the reinforcer, animals were re-exposed to predictable or unpredictable footshock and were allowed to respond for the fentanyl solution. Again, females self-administered significantly more fentanyl than did the males during this relapse phase. However, the effect for predictability was not there. This finding indicated that predictability might play an important role in mediating drug self-administration during maintenance of drug-taking behavior, but that it plays a less important role in drug relapse. These animal data are consistent with human reports that predictability has beneficial effects (e.g., increased performance and persistence on cognitive tasks) under acute conditions compared to unpredictable stress (e.g., Glass & Singer, 1972), but that exposure to a chronic, predictable stressor might have greater stress effects (i.e., biochemical & behavioral) than would an unpredictable stressor (Abbott, Schoen, & Badia, 1984; Arthur, 1986; McKinnon, Weisse, Reynolds, Bowles, & Baum, 1989).

Next, a study was designed to examine sex differences in sensitivity to footshock stress and the blockade of endogenous opioid peptides to examine whether or not sex differences in fentanyl self-administration are a result of differential sensitivity to the shock stimulus (Popke, Klein, Alvares, & Grunberg, 1994). The behavioral data indicate that males and females are equally stressed by this shock amplitude and that the differences in

drug self-administration are not a result of sex differences in stressor sensitivity.

The final study in this series was designed to examine the effects of a non-painful, social stressor (crowded versus individually housed conditions) on fentanyl consumption in male and female rats (Brown, Klein, Rahman, & Grunberg, 1995). Based on Brown and Grunberg (1995), it was hypothesized that individually housed females would be more stressed than would crowded females (as indexed by plasma corticosterone), but that crowded males would be more stressed than would individually-housed males. Using the home cage oral opioid self-administration paradigm from Shaham et al. (1993), this experiment found that non-stressed females (i.e., crowded) consumed more fentanyl than did stressed females (i.e., individually housed) and that both groups of females self-administered more fentanyl than did stressed and non-stressed males. This gender difference in opioid self-administration is consistent with Alexander and colleagues (Alexander et al., 1981; Alexander et al., 1978) and Klein et al. (1997). Along with other recent experiments with adult male rats (Shaham, 1993; Shaham & Stewart, 1994, 1995), these results indicate that a causal relationship exists between stress and opiate self-administration that is not related to the analgesic properties of these drugs, that this effect of stress occurs across different types of stressors, and that sex differences in opioid consumption exist. It is noteworthy that all of these studies used only adult animals.

As discussed earlier, no studies on the gateway hypothesis have evaluated the role that stress may play in affecting opioid consumption. However, in light of human and animal reports that stress influences drug consumption and that there are possible sex differences in these effects, the present experiment also examined the effects of stress on

opioid consumption in adult male and female rats with and without a history of nicotine exposure. It is noteworthy that, like nicotine and opioid administration, stress activates the ventral tegmental area (i.e., dopaminergic pathway activation) and may be a mechanism by which stress is related to opioid use (Grunberg, 1994). Specifically, half of the animals were exposed to immobilization stress every day prior to access to an opioid solution. Reports indicate that male and female rats repeatedly exposed to this stressor (at least 14 days) display increases in biochemical (e.g., corticosterone, ACTH, prolactin) indicative of a stress response, regardless of opioid consumption (Kant et al., 1983; Kant, Leu, Anderson, & Mougey, 1987; Raygada, Shaham, Nespor, Kant, & Grunberg, 1992; Shaham et al., 1992). These findings suggest that biochemical responses of rats do not habituate following repeated exposure to this physical stressor and, therefore, it could be used in the present experiment.

OVERVIEW

The purpose of the present experiment was to examine the effects of nicotine administration during adolescence on subsequent opioid self-administration (SA) in male and female rats. The present experiment also examined the effects of nicotine administration on the subsequent effects of stress and no-stress on opioid SA using a 3 (nicotine) x 2 (sex) x 2 (stress) experimental design (see Table 1). Specifically, 41-day-old rats received saline (n = 40), 6 mg of nicotine /kg/day (n = 40), or 12 mg of nicotine/kg/day (n = 40) by osmotic minipump for 24 hours/day for 19 days. Then, following a 7-day drug cessation period, rats were provided fentanyl solution in their home cages and drug consumption was evaluated every day for 4 weeks. Throughout the opioid self-administration phase of the experiment, rats received either 20 minutes of immobilization stress each day (n = 60) or no-stress (n = 60) prior to the opioid availability. In addition to drug consumption, body weight and food and water consumption were measured throughout the experiment. For males, it was hypothesized that nicotine exposure would increase subsequent opioid self-administration in a positive, linear, dose-dependent manner. For females, it was hypothesized that nicotine exposure would be related to subsequent opioid self-administration by an inverted-U shaped function. With respect to the effects of stress, it also was hypothesized that immobilization stress would: increase opioid SA by males compared with non-stressed males; and decrease opioid SA by females compared with non-stressed females. It also was hypothesized that female rats would SA more opioid solution than would male rats, regardless of previous nicotine exposure.

HYPOTHESES

There were two major, original hypotheses and six additional hypotheses that were based on predictions to replicate and extend previous findings. Major Hypothesis 1 addresses the gateway hypothesis. Major Hypothesis 2 addresses the relationship between stress and drug self-administration.

Major Hypotheses

Major Hypothesis 1: It was hypothesized that nicotine exposure would increase subsequent fentanyl self-administration and that the dose-effects would differ in male and female rats. Specifically, male rats previously exposed to saline would self-administer lower amounts of fentanyl than would male rats previously exposed to 6 mg nicotine/kg/day and that both of these groups of animals would self-administer lower amounts of fentanyl than would male rats previously exposed to 12 mg nicotine/kg/day. Female rats previously exposed to 6 mg nicotine/kg/day would self-administer more fentanyl than would female rats previously exposed to saline or to 12 mg nicotine/kg/day.

Rationale: The gateway hypothesis, based on epidemiologic data, holds that cigarette smoking precedes self-administration of illicit drugs, including opioids (Blaze-Temple & Lo, 1992; Kandel, 1975; Kandel et al., 1978; Kandel, & Yamaguchi, 1985; Kandel & Yamaguchi, 1993; Kandel et al., 1992; Newcomb & Bentler, 1986). Because nicotine is the primary active pharmacologic agent in tobacco, it was hypothesized that nicotine exposure would result in subsequent increases in opioid self-administration. The linear dose-response prediction follows from reports that males respond in a linear dose-response fashion to various effects of nicotine administered via osmotic minipump across

the dose range used in the present experiment (Winders & Grunberg, 1989). An inverted U-shaped curve was predicted for females because females may be more sensitive to the effects of nicotine (Bättig et al., 1982; Silverstein et al., 1980; Rosecrans, 1971; Rosecrans, 1972; Grunberg et al., 1991) and nicotine, reportedly, has stimulatory and then depressive effects as the dosage increases (USDHHS, 1988; Volle & Koell, 1975).

Major Hypothesis 2: It was hypothesized that immobilization stress would alter opioid self-administration. Specifically, stress would increase opioid self-administration by male rats compared with non-stressed male rats, regardless of prior nicotine or saline exposure. In contrast, immobilization stress would decrease opioid self-administration by female rats compared with non-stressed female rats, regardless of previous nicotine or saline exposure.

Rationale: Previous investigations (Shaham et al., 1992; Shaham et al., 1993) report that stress (e.g., footshock, immobilization) increases opioid self-administration by male rats compared with no-stress control conditions. Brown, Klein, Rahman, and Grunberg (1995) reported that non-stressed (i.e., crowded housing conditions) female rats self-administer significantly more fentanyl than do stressed female rats.

Hypotheses Made To Replicate and Extend Previous Reports

Hypothesis 1 addresses predicted differences between males and females in opioid self-administration. Hypotheses 2 and 3 address predictions regarding opioid withdrawal. Hypothesis 4 addresses stress, sex, and plasma corticosterone. Hypotheses 5 and 6 address predictions regarding nicotine and body weight.

Hypothesis 1: It was hypothesized that female rats would self-administer more

opioid solution than would male rats, regardless of previous nicotine exposure.

Rationale: Previous reports indicate that female rats self-administer significantly more fentanyl than do male rats, regardless of physical stressor (Klein et al., 1997) or social crowding (Alexander, Coambs, & Hadaway, 1978; Brown et al., 1995) conditions. Therefore, female rats exposed to nicotine or saline should self-administer greater amounts of the fentanyl solution than would male rats exposed to nicotine or saline.

Hypothesis 2: It was hypothesized that male rats would exhibit greater opioid withdrawal behaviors in response to opioid antagonism than would female rats despite lower amounts of fentanyl self-administration by male rats.

Rationale: Klein et al. (1997) reported that male rats exposed to stress (i.e., footshock) and fentanyl solution exhibited greater opioid withdrawal behaviors in response to naloxone challenge than did female rats, despite lower amounts of fentanyl SA by male rats.

Hypothesis 3: It was hypothesized that male rats exposed to immobilization stress would exhibit a greater number of withdrawal behaviors in response to naloxone challenge (i.e., opioid antagonism) than would male rats not exposed to stress, female rats exposed to immobilization stress, and female rats not exposed to stress.

Rationale: Popke, Klein, Alvares, and Grunberg (1994) reported that naloxone injection following footshock stress increases freezing (an index of stress) by male rats but not by female rats.

Hypothesis 4: It was hypothesized that rats exposed to immobilization stress would have higher levels of plasma corticosterone than would rats that were not

immobilized. In addition, it was hypothesized that female rats exposed to immobilization stress would have greater levels of plasma corticosterone than would male rats exposed to immobilization stress.

Rationale: Previous studies indicate that animals repeatedly exposed to immobilization stress (at least 14 days), regardless of opioid self-administration, have higher plasma corticosterone, adrenocorticotrophin hormone (ACTH), and prolactin levels than do animals not exposed to stress (Kant et al., 1983; Kant et al., 1987; Raygada et al., 1992; Shaham et al., 1992). Also, stressed female rats have greater levels of plasma corticosterone than do stressed male rats (Brown & Grunberg, 1995; Kant et al., 1983; Klein et al., 1997).

Hypothesis 5. It was hypothesized that rats exposed to nicotine would gain less weight during the nicotine exposure phase of the experiment than would rats exposed to saline and that this weight difference would be greater in female rats.

Rationale: It is well-established that nicotine administration is inversely related to body weight and that the effect is greater for females (Grunberg, 1992; Winders & Grunberg, 1989).

Hypothesis 6. It was hypothesized that rats exposed to nicotine would gain more weight during the nicotine abstinence phase of the experiment than would animals exposed to saline and that this weight difference would be greater in female rats.

Rationale: It is well-established that nicotine cessation results in significant weight gain and that this effect is greater for females (Grunberg, 1992; Winders & Grunberg, 1989).

METHODS

Subjects

Subjects were 60 female and 60 male Wistar rats (Charles River Laboratories, Wilmington, MA). Wistar rats were the subjects because they consume fentanyl solution in various laboratory settings similar to the present experiment (Brown et al., 1995; Klein et al., 1997; Klein et al., 1993; Shaham et al., 1992; Shaham, 1993). The sample size was based on empirical investigations that used similar self-administration paradigms and reported statistically significant findings with sample sizes per cell of 10 animals or less (Brown et al., 1995; Klein et al., 1997; Klein et al., 1993; Shaham et al., 1992; Shaham, 1993). All rats were approximately 33 days old and weighed 50-75 g at the beginning of the experiment. This age was selected to ensure that subjects were premature ("adolescent") during nicotine or saline administration (42-60 days old) and that subjects were young adults (66-99 days old) during the opioid self-administration phases (i.e., initiation, maintenance) of the experiment. In addition, significant results have been reported on behavioral responses (e.g., acoustic startle response (ASR) and inhibition of ASR) with nicotine administration in male rats in this age range (39-42 days) (Acri, Brown, Saah, & Grunberg, 1995). Wistar rats are considered sexually mature at 8-10 weeks (females) or 10-12 weeks (males) of age at which time they weigh 180-200 g (females) or 350-400 g (males). Animals were individually housed in standard shoebox cages (35.6 cm x 15.2 cm x 20.3 cm) on absorbent cellulosic fiber contact bedding (Cell-Sorb Plus™) during the nicotine exposure phase of the experiment and on absorbent hardwood chip contact bedding (Pine-Dri) at all other times. The animal room was

maintained at 23° C, 50% relative humidity, on a 12-hour light/dark cycle (lights on at 0700 hours). Throughout the experiment, animals had continuous access to standard laboratory food pellets (Harlan Teklad 4% Mouse/Rat Diet 7001) through a stainless steel wire-bar lid with slotted feeders. Tap water was used as the water source and to make the fentanyl solution.

Drugs

Nicotine dihydrochloride. Nicotine dihydrochloride solution (dissolved in physiological saline) or physiological saline (control) was administered using Alzet mini-osmotic pumps (see Figure 3; Model 2002, Alza Corporation). Minipumps were filled with nicotine solution (see Appendix I) or saline and delivered the solution at a rate of 0.5 µl/hour (Theeuwes & Yum, 1977). Dosages of 12 mg nicotine base/kg/day, 6 mg nicotine base/kg/day, or 0 mg/kg/day were used. These nicotine dosages were selected on the basis of previous studies of nicotine and body weight, food consumption, behavioral responses (e.g., acoustic startle response) and biochemical responses (e.g., insulin, glucose, catecholamines) (Acri et al., 1995; Acri, Grunberg, & Morse, 1991; Grunberg et al., 1985; Grunberg et al., 1988; Grunberg, Winders, & Popp, 1987).

This drug administration paradigm and these doses have been used extensively in rats and have been reported to yield results that are comparable with effects of smoking by humans (Acri et al., 1995; Acri et al., 1991; Grunberg, 1982; Grunberg et al., 1984; Grunberg, 1992; Winders & Grunberg, 1989). The average cigarette smoker smokes about 1-2 packs or 20-40 cigarettes a day. Pharmacokinetic studies suggest that 1-2 pack/day smokers have venous plasma nicotine levels of about 50 ng/ml and arterial

plasma levels of approximately 100-150 ng of nicotine/ml (Benowitz, 1987). Investigations with rats have used trunk blood which contains mostly arterial blood. Published reports with male rats suggest that the 6 mg nicotine/kg/day minipump preparation yields plasma nicotine levels of approximately 100 ng/ml, similar to the average human smoker (Richardson & Tizabi, 1994). Other pharmacokinetic studies indicate that exposure to 12 mg nicotine/kg/day via minipump results in plasma nicotine levels of about 450 ng/ml (range: 200-900 ng/ml) in male rats (as assayed by N. Benowitz; N. Grunberg, personal communication, March, 1996). This plasma value is higher than that found in the average smoker. However, it has been suggested that a ratio of 8:1 should be taken into consideration when comparing drug plasma levels in rats with that found in humans (A. Alvares, personal communication, April, 1996). Examination of possible gender differences in the pharmacokinetics of nicotine suggests that male smokers metabolize and excrete nicotine more rapidly than do female smokers (Benowitz, Kuyt, & Jacob, 1984). The present experiment included both nicotine dosages because of studies of nicotine in rats that have yielded behavioral (e.g., food consumption, acoustic startle response) and biochemical responses (e.g., catecholamines) that are similar to effects of nicotine in humans (Acri et al., 1995; Acri et al., 1991; Gritz et al., 1989; Grunberg, 1992; Grunberg et al., 1985; Grunberg et al., 1988; Grunberg et al., 1987; Klesges & Klesges, 1988; Klesges et al., 1989).

Fentanyl hydrochloride. Fentanyl hydrochloride (HCl) (Mallinckrodt Chemicals, Inc), in a concentration of 50 µg/ml dissolved in tap water was used. Previous studies report that adult female and male rats self-administer this concentration of fentanyl-HCl

solution orally (Shaham et al., 1993; Klein et al., 1993; Klein et al., 1997). A recent report suggests that immobilization stress does not alter fentanyl metabolism in male Wistar rats (Cheriathundam, Shaham, Klein, Grunberg, & Alvares, 1996).

Naloxone hydrochloride. Opioid withdrawal syndrome was precipitated by intraperitoneal (IP) injection of 1.5 mg/kg naloxone-HCl (DuPont Pharmaceutical) based on the procedures of Shaham et al. (1993), Popke et al. (1994), and Klein et al. (1997). Naloxone-HCl, suspended in 0.86% NaCl solution in a concentration of 0.4 mg/ml, was used.

Stress Manipulation

Animals in the stress condition (n = 60) were restrained in Centrap restraint cages (Fischer Scientific) for a period of 20 minutes based on the procedures of Raygada et al. (1992) and Shaham et al. (1992). Animals were placed in the finger-like apparatus and the cage was tightened until the animal's movements are restricted but not enough to cause pinching or pain. Earlier reports indicate that repeated exposure to this restraint procedure for at least 14 days, with or without concurrent opioid administration, results in a reliable increase in biochemical responses thought to be indicative of a stress response (e.g., corticosterone, ACTH, prolactin) (Kant et al., 1983; Kant, Leu, Anderson, & Mougey, 1987; Raygada et al., 1992; Shaham et al., 1992).

Procedure

Table 2 presents the timeline of the experiment and the associated ages of the rats during each phase of the experiment. The experiment was conducted in seven phases: (1) baseline; (2) nicotine or saline exposure; (3) cessation of nicotine or saline; (4) opioid

initiation; (5) opioid maintenance; (6) opioid abstinence; and (7) measurement of corticosterone. Animals were exposed to either immobilization stress (IM) or no-stress during the two phases of the experiment with opioid availability (i.e., phases 4 and 5). Prior to the baseline phase, animals were gentled for approximately 5 minutes a day for 3 days. Body weight, food and water consumption were measured and recorded throughout the experiment.

Baseline. Rats were handled and food and water consumption and body weight were measured daily. After the 5-day baseline period (days 4-8), subjects were assigned to experimental groups based on food and water consumption and body weight to ensure that the treatment groups within each sex did not differ significantly from one another.

Nicotine or saline exposure. Following baseline, osmotic minipumps containing either saline or nicotine were implanted (day 9) into all subjects based on procedures reported in the literature and used in this laboratory (Acri et al., 1995; Acri et al., 1991; Grunberg, 1982; Grunberg et al., 1984; Grunberg, Popp, & Winders, 1988; Grunberg et al., 1987). Specifically, animals were anesthetized by exposure to a methoxyflurane-soaked (Metophane®, Pitman-Moore, Inc.) gauze pad in a closed bell-jar within a vented fume hood. Next, a 4 x 4 cm area between the withers was shaved, sterilized with betadine, and a 2 cm mid-line horizontal incision was made approximately 1 cm below the scapulae with blunt-nosed, curved-tipped Mayo surgical scissors (Roboz® Surgical Instruments). Then, the scissors were inserted into the incision, cephalad, to make a small subcutaneous (SC) pocket. Finally, a minipump containing the appropriate nicotine or saline solution was implanted into the SC pocket with the one-way release valve pointed

cephalad. Each incision was closed with 9 mm stainless steel wound clips (MikRon® AUTOCLIP®, Becton Dickinson & Company) and then the animal was returned to its home cage. The entire implant procedure took less than 3 minutes per animal. Animals were weighed and food and water consumption were evaluated daily for 19 days (days 10-28).

Nicotine or saline abstinence. On the last minipump day (day 28), animals were anesthetized following the procedure described above and the minipumps were explanted. Specifically, a new 2 cm mid-line horizontal incision was made 1-2 cm above the original incision, the pump was removed manually, and the incision was closed with 9 mm stainless steel wound clips. Animals were kept in their home cages for seven additional days to allow for nicotine withdrawal prior to the opioid phase of the experiment. This withdrawal period was included to insure that any differences in opioid consumption were not a result of acute nicotine withdrawal, such as irritability. Food and water consumption were measured on six of the seven days (days 29-35).

Opioid initiation. Next, fentanyl solution was made available to all subjects to examine drug self-administration. On each day, animals had access to fentanyl solution alone (FO) or they had access to the opioid solution and tap water (choice [CH]). FO days and CH days were cycled every 5 days with 1 CH day and 4 FO days. The FO days were included to increase the likelihood that dependence on fentanyl would develop and is based on the methods used in prior oral fentanyl SA studies with adult males (Shaham et al., 1992). The first 3 days of initiation (days 36-38) were CH days in order to examine initial preference (or choice) for the fentanyl solution. The fentanyl solution, with or

without water, was made available in the home cage for 6 hours a day between 0900 to 1800 hours (see Appendix II for schedule of opioid availability). Food also was available during the 6-hour drug availability period. Food and only tap water were available for the remaining 18 hours a day. This schedule of opioid solution availability was based on previous published reports with morphine and fentanyl solution in adult rats (Brown et al, 1995; Shaham, 1993; Shaham et al., 1992). The position of the drug and water bottles were switched daily to decrease the likelihood of conditioned place preferences. Opioid initiation was evaluated during the 8 days (days 36-43) of initial exposure to the fentanyl solution.

In addition to exposing subjects to the fentanyl solution, the stress manipulation (IM) occurred every day (beginning on day 36), 20 minutes prior to the fentanyl access period. Animals in the IM condition (n = 60) were restrained in a separate treatment room, in same-sex groups of 10 that included at least 2 animals from the saline, low-nicotine, and high-nicotine treatment groups. Following the stressor, animals were returned to their home cages where the drug was made available. Order of IM exposure alternated between males and females and was rotated every day (see Appendix II for stressor schedule). Non-stressed animals (n = 60) were left in their home cages in the colony room during this time and received the fentanyl solution in groups of 10 when the matched stressed animals were returned to their home cages. Throughout this initiation phase, fentanyl and water consumption, body weight, and food consumption were measured daily.

Opioid maintenance. Fentanyl access continued in 5-day cycles as described for

the opioid initiation phase of the experiment and ended with an additional CH day. This maintenance phase lasted a total of 21 days (i.e., 4 cycles plus CH day; days 44-64). Next, subjects were exposed to two additional CH days (days 65-66), including IM or no-IM conditions, in order to evaluate opioid withdrawal behaviors. Specifically, naloxone-HCl (1.5 mg/kg) was injected IP into subjects following the 6-hour consumption period and subjects were returned to their home cages. Half of the subjects (i.e., 60 subjects; 5 from each experimental group) received naloxone injections following fentanyl access on day 65 and the second group of subjects received naloxone following fentanyl access on day 66 (see Appendix III for naloxone treatment schedule). Opioid withdrawal behaviors (i.e., wet-dog shakes, diarrhea, mouthing and teeth chattering, ptosis, excessive grooming, abnormal posture) were evaluated by two trained independent observers (inter-rater reliability coefficient = +0.90). Behavioral assessment began 5 minutes after injection and lasted for 20 minutes (see Appendix IV for a copy of the naloxone withdrawal observation data sheet). This procedure is based on reports by Klein et al. (1997) and others (Shaham, 1993; Shaham et al., 1993; Linseman, 1977) and has reported an inter-rater reliability coefficient of +0.96 (Pearson's product-moment correlation) (Klein et al, 1997; Popke et al., 1994). IM or no-IM conditions were conducted every day prior to access to the fentanyl solution. In addition, fentanyl and water consumption, body weight, and food consumption were measured daily.

Opioid abstinence. Following the last day of withdrawal assessment, the fentanyl solution no longer was available to the animals and subjects had access only to tap water and food. No stress manipulation occurred and body weight, food and water consumption

were measured for five days (days 67-71).

Measurement of corticosterone. On the last day of the experiment (day 72), animals in the IM group were exposed to the 20-minute stressor. Within 15 minutes of the cessation of the stressor, all subjects (stress and no-stress) were decapitated without anesthesia based on the procedures of Klein et al. (1997) and others (Raygada et al., 1992; Shaham et al., 1993; Brown & Grunberg, 1995). Trunk blood was collected in 7 ml collection tubes (Vacutainer®) that were treated with 0.07 ml of 15% ethylenediamine tetra-acetic acid (EDTA; K_3) solution (10.5 mg). Samples immediately were placed on ice and then were centrifuged (1500 x g) for 20 minutes at 4° C. Approximately 3000 µl of plasma was stored and frozen at -70° C in separate micro-tubes for later measurement of corticosterone by radioimmunoassay (RIA; ICN Biomedical; see Appendix V for assay procedures).

Statistical Analyses

Overall, this experiment used a 3 (nicotine) x 2 (sex) x 2 (stress) between-subjects design to examine the effects of nicotine administration on subsequent fentanyl consumption and the effects of immobilization stress on fentanyl consumption in male and females rats. The between-subjects dependent variables that were evaluated by univariate and multivariate analysis of variance (ANOVA) were: body weight, food and water consumption, fentanyl consumption, water consumption on fentanyl/water choice days, percent fentanyl consumption (i.e., fentanyl preference) on fentanyl/water choice days, withdrawal scores following naloxone challenge, and plasma corticosterone levels.

At the end of the baseline phase, food and water consumption and body weight

were averaged across the 5 baseline days. Then, a series of ANOVAs were used on these three variables to assign subjects to experimental groups based on food and water consumption and body weight to insure that experimental groups within each sex did not differ significantly from one another. This statistical approach also was used to examine the effects of nicotine exposure on body weight, food, and water consumption during the nicotine exposure and nicotine cessation phases of the experiment. Specifically, food and water consumption and body weight were averaged across the 19 nicotine or saline exposure days and also were averaged across the 6 measurement days during the nicotine or saline cessation phase. A series of 3-way ANOVAs were used separately on these three variables to examine effects of nicotine exposure.

In order to make comparisons between males and females in the different treatment conditions, fentanyl consumption was adjusted for body weight and fentanyl amounts were calculated for each animal for each day of fentanyl exposure. Specifically, the volume of fentanyl solution consumed (ml) for each day of each fentanyl phase of the experiment (i.e., opioid initiation, opioid maintenance) was multiplied by the concentration of the fentanyl solution (0.05 mg fentanyl HCl/ml water). This product then was divided by the subject's body weight (kg) for that particular day such that:

$$\frac{\text{(volume fentanyl solution consumed [ml])} \times \text{(0.05 mg fentanyl HCl/ml } H_2O)}{\text{body weight of subject (kg)}}$$

yielded the total amount of fentanyl consumption for each subject (mg/kg). Next, fentanyl consumption data were averaged across the total number of days for each fentanyl phase of the experiment. To evaluate differences in drug initiation and maintenance, amount of

fentanyl consumption (mg/kg) was analyzed by ANOVA for each drug phase of the experiment (i.e., initiation, maintenance). Major Hypothesis 1 was analyzed by a 3 (nicotine) x 2 (sex) ANOVA and Major Hypothesis 2 was analyzed by a 2 (sex) x 2 (stress) ANOVA for each drug phase of the experiment. Confirmatory Hypothesis 1 was tested using a univariate ANOVA with two levels of sex as the independent variable.

The fentanyl preference data for each choice day were averaged across the total number of choice days for each drug phase of the experiment. Next, differences in fentanyl preference during drug initiation and maintenance were examined. Proportion of fentanyl consumption on each choice day was calculated for each subject by adding the volume of fentanyl solution consumed (ml) to the volume of water solution consumed (ml) during each 6-hour period to determine total volume consumption (ml). Next, the volume of fentanyl consumed (ml) was divided by the total amount of liquid consumed during that same time period (ml) and this product was multiplied by 100 to calculate the percent of fentanyl preference for each fentanyl-water choice session. Or:

$$\text{\% fentanyl preference} = \frac{\text{volume fentanyl solution consumed [ml]}}{\text{(fentanyl solution consumed [ml]) + (water consumed [ml])}} \times 100$$

These data were analyzed following the same strategy as described for the fentanyl consumption data.

In order to evaluate differences in opioid withdrawal (Confirmatory Hypotheses 2 and 3), the number of occurrences of withdrawal symptoms observed by both raters during the 20-minute observation period were added to determine a total withdrawal score for each subject. These scores were analyzed by a two-way ANOVA with two levels of

sex and two levels of stress as the independent variables. Differences in plasma corticosterone levels (Confirmatory Hypothesis 4) also were tested by a two-way ANOVA with sex and stress as the independent variables. The effects of nicotine and nicotine cessation on body weight (Confirmatory Hypotheses 5 and 6) were evaluated separately for males and females by one-way ANOVAs with three levels for nicotine dosage (0 mg/kg/day, 6 mg nicotine/kg/day, and 12 mg nicotine/kg/day).

The within-subject analyses for this experiment included the 12 experimental groups (3 [nicotine] x 2 [sex] x 2 [stress]) evaluated during drug initiation. Repeated-measures ANOVAs were used to evaluate the effects of nicotine, sex, and stress on the rate of drug initiation with amount of fentanyl consumption (mg/kg) as the dependent variable.

Regression analyses were used to evaluate: (1) whether or not sex, stress (as indexed by plasma corticosterone), and nicotine pre-exposure predict subsequent fentanyl consumption; and (2) the relationship between fentanyl consumption during initiation and maintenance and withdrawal responses following naloxone challenge. All significance tests were two-tailed and were evaluated at an alpha level of 0.05. Tukey HSD *post-hoc* analyses ($\alpha = 0.05$) were conducted where appropriate.

RESULTS

Overview

The results are presented in the chronological order of the experiment: Baseline, Nicotine or Saline Exposure, Nicotine or Saline Abstinence, Opioid Initiation, Opioid Maintenance, Opioid Withdrawal Assessment, Opioid Abstinence, and Corticosterone. Within most of these sections (with the exception of the Opioid Withdrawal Assessment and Corticosterone sections), body weight, food consumption, and water consumption results are presented first. For the Opioid Initiation and Opioid Maintenance phases, opioid consumption and fentanyl preference are presented next. The Opioid Withdrawal Assessment and Corticosterone results sections present the results of the variables examined. The results relevant to the two Major Hypotheses appear under *Opioid Consumption* in the **Opioid Initiation** (p. 54) and **Opioid Maintenance** (p. 57) results sections.

Two female subjects in the no-stress, 12 mg nicotine/kg/day group were not included in the analyses subsequent to the opioid initiation phase because they died of apparent drug overdose (i.e., respiratory distress and failure) on the first fentanyl-only day of the experiment (i.e., day 5 of fentanyl exposure).

Baseline

During this phase of the experiment, rats were handled and food and water consumption and body weight were measured and recorded for 5 days. Body weights, food, and water consumption were averaged across the 5-day baseline period. Three-way analyses of variance (ANOVAs) were conducted separately for each of these variables to

assign subjects to experimental groups based on food and water consumption and body weight. Sex (2), stress (2), and drug condition (3) were the independent variables. Two-way ANOVAs were conducted on these variables with stress (2) and drug condition (3) as the independent variables to ensure that experimental groups within each sex did not differ significantly from one another.

Body weight. Figure 4 presents mean baseline body weights (grams) for all 12 treatment groups for each phase of the experiment. Three-way ANOVA indicated that there was a significant main effect for sex with males weighing more than females during baseline [$F(1,108) = 115.09$, $p < .05$]. There were no significant baseline body weight differences among the subjects that became the different drug treatment groups (i.e., 0, 6, or 12 mg nicotine/kg/day) and there were no significant baseline differences in body weight among the subjects that later became the stress or no-stress groups. There also were no significant baseline 2- or 3-way interactions. See Table 3 for a complete list of statistical values. Separate 2-way ANOVAs for males and females did not reveal significant main effects or a 2-way interaction for drug condition or stress at baseline.

Food consumption. Figure 5 presents mean food consumption (grams) for all 12 treatment groups for each phase of the experiment. Three-way ANOVA indicated that there was a significant main effect for sex with males eating more food than females [$F(1,108) = 33.83$, $p < .05$]. There were no significant baseline differences in food consumption among the subjects that became the different drug treatment groups (i.e., 0, 6, or 12 mg nicotine/kg/day) and there were no significant baseline differences in food consumption among the subjects that later became the stress or no-stress groups. There

also were no significant 2- or 3-way interactions at baseline. See Table 4 for a complete list of statistical values. Separate 2-way ANOVAs for males and females did not reveal significant main effects or a 2-way interaction for drug condition or stress at baseline.

Water consumption. Figure 6 presents mean water consumption (ml) for all 12 treatment groups for each phase of the experiment. Three-way ANOVA indicated that there were no significant baseline differences in water consumption between male and female rats. In addition, there were no significant baseline differences in water consumption among the subjects that became the different drug treatment groups (i.e., 0, 6, or 12 mg nicotine/kg/day). However, there was a significant main effect for stress with animals that later were stressed during the opioid phase of the experiment consuming more water, on average, than did the animals that were not stressed later [$F(1,108) = 4.25$, $p < .05$]. There were no significant 2- or 3-way interactions at baseline. See Table 5 for a complete list of statistical values. Separate 2-way ANOVAs for males and females did not reveal a significant main effect for drug condition or a 2-way interaction at baseline. Among males, there was no significant difference in water consumption between stress conditions. However, female rats that later were stressed during the opioid phase of the experiment consumed more water during the baseline phase than did the female rats that were not stressed later [$F(1,54) = 7.64$, $p < .05$].

Nicotine or Saline Exposure

Following nicotine (6 mg/kg/day or 12 mg/kg/day) or saline minipump implantation, rats were handled and food and water consumption and body weight were measured and recorded for 19 days. Body weights, food, and water consumption were

averaged across the 19-day baseline period. Three-way analyses of variance (ANOVAs) were conducted separately for each of these variables with sex (2), stress (2), and drug condition (3) used as the independent variables. One-way ANOVAs were conducted separately for males and females to determine significant effects of nicotine on body weight and food consumption with drug condition (3) as the independent variable.

Body weight. Analysis of body weights collapsed within each of the 12 treatment groups across this phase indicated that there was a significant main effect for sex with males weighing more than females [$F(1,108) = 542.76$, $p < .05$] (see Figure 4). There also was a significant main effect for drug condition [$F(2,108) = 9.35$, $p < .05$]. Separate one-way ANOVAs for males and females revealed significant main effects for drug condition [$F(2,57) = 3.62$, $p < .05$ and $F(2,57) = 7.30$, $p < .05$, respectively]. To further examine the significant effects of nicotine exposure on body weight, Tukey HSD *post-hoc* analyses were conducted separately for males and females. These results indicated that male rats exposed to 12 mg nicotine/kg/day weighed significantly less than did male rats administered saline (235.65 ± 14.25 g versus 249.33 ± 19.22 g). Among females, subjects exposed to 12 mg nicotine /kg/day weighed less than did subjects exposed to saline (175.46 ± 10.42 g versus 189.33 ± 12.06 g). There were no significant differences in body weight among the subjects that later became the stress or no-stress groups and there were no significant 2- or 3-way interactions. These results are listed in Table 6.

Because there was a significant main effect for sex on body weight during baseline, body weight during nicotine or saline exposure was analyzed with a 3-way analysis of covariance (ANCOVA), using baseline body weight as a covariate and taking into account

all main effects, 2-way, and 3-way interactions. The significant main effects for sex [$F(1,107) = 285.18$, $p < .05$] and for drug condition [$F(2,107) = 27.19$, $p < .05$] still held with females weighing less than males and nicotine-treated subjects weighing less than saline-treated subjects. There were no other significant main effects or interactions.

Food consumption. Analysis of food consumption collapsed within each of the 12 treatment groups across this phase indicated that there was a significant main effect for sex with males consuming more food than did females [$F(1,108) = 366.53$, $p < .05$] (see Figure 5). There also was a significant main effect for drug condition [$F(2,108) = 18.07$, $p < .05$] with nicotine-treated subjects eating less food. Separate one-way ANOVAs for males and females revealed significant main effects for drug condition [$F(2,57) = 6.84$, $p < .05$ and $F(2,57) = 14.92$, $p < .05$, respectively]. Tukey HSD *post-hoc* analysis indicated that male rats exposed to 12 mg nicotine/kg/day ate significantly less food than did male rats administered saline (27.89 ± 2.09 g of food versus 30.70 ± 2.88 g of food). Tukey HSD *post-hoc* analysis indicated that, among females, both the 12 and 6 mg nicotine/kg/day doses produced significant decreases in food consumption compared with the saline condition (20.84 ± 1.59 and 21.88 ± 1.64 g of food versus 23.63 ± 1.67 g of food). There were no significant differences in food consumption among the subjects that later became the stress or no-stress groups and there were no significant 2- or 3-way interactions. These results are listed in Table 7.

Because there was a significant main effect for sex on food consumption during baseline, food consumption during nicotine or saline exposure was analyzed with a 3-way analysis of covariance (ANCOVA), using baseline food consumption as a covariate and

taking into account all main effects, 2-way, and 3-way interactions. The significant main effects for sex [$F(1,107) = 251.12$, $p < .05$] and for drug condition [$F(2,107) = 21.43$, $p < .05$] still held with females consuming less food than males and nicotine-treated subjects consuming less food than saline-treated subjects. There were no other significant main effects or interactions.

Water consumption. Analysis of water consumption collapsed within each of the 12 treatment groups across this phase indicated that there was a significant main effect for sex with males drinking more water than did females [$F(1,108) = 32.84$, $p < .05$] (see Figure 6). There also was a significant main effect for stress condition [$F(1,108) = 9.62$, $p < .05$] with subjects that later became the stress group drinking more water than did subjects in the no-stress groups. There was no main effect for drug condition and there were no significant 2- or 3-way interactions. These results are listed in Table 8. Separate 2-way ANOVAs for males and females did not reveal a significant main effect for drug condition or a 2-way interaction. Among males, there was no significant difference in water consumption between stress and no-stress conditions [33.00 ± 0.80 ml versus 32.85 ± 0.72 ml of water; $F(1,54) = 2.20$, n.s.]. However, female rats that later were stressed during the opioid phase of the experiment consumed more water during the nicotine or saline exposure phase than did the female rats that were not stressed later [33.72 ± 0.89 ml versus 30.63 ± 0.65 ml of water; $F(1,54) = 8.51$, $p < .05$].

Nicotine or Saline Abstinence

Following 19 days of nicotine or saline exposure, minipumps were explanted and body weight, food and water consumption were evaluated for 7 days to examine effects of

nicotine withdrawal. Body weights, food, and water consumption were averaged across 6 days of the 7-day cessation period. Three-way analyses of variance (ANOVAs) were conducted separately for each of these variables with sex (2), stress (2), and drug condition (3) used as the independent variables. One-way ANOVAs were conducted separately for males and females to determine significant effects of nicotine on these dependent variables with drug condition (3) as the independent variable.

Body weight. Analysis of body weights collapsed within each of the 12 treatment groups across this phase indicated that there was a significant main effect for sex with males weighing more than females [$F(1,108) = 932.20$, $p < .05$] (see Figure 4). There also was a significant main effect for drug condition [$F(2,108) = 3.52$, $p < .05$]. Separate one-way ANOVAs for males and females revealed a significant main effect for drug condition for females, but not for males [$F(2,57) = 3.49$, $p < .05$ and $F(2,57) = 1.16$, n.s., respectively]. Tukey HSD *post-hoc* analyses indicated that female rats exposed to 12 mg nicotine/kg/day continued to weigh significantly less than did female rats administered saline (217.02 ± 2.83 g versus 228.85 ± 3.13 g). There were no significant differences in body weight among the subjects that later became the stress or no-stress groups and there were no significant 2- or 3-way interactions. These results are listed in Table 9.

In order to examine changes in body weight during nicotine cessation, mean body weight for the first 2 days (i.e., days 1-2 of cessation), the next 2 days (i.e., days 3-4 of cessation), and last 2 days (i.e., days 6-7 of cessation) of nicotine or saline cessation were analyzed separately by 3-way ANOVAs. There was a significant main effect for sex [$F(1,108) = 916.57$, $p < .05$] and for drug condition [$F(2,108) = 4.33$, $p < .05$] during the

first 2 days of nicotine or saline cessation and there were no other significant main effects or interactions. Separate one-way ANOVAs for males and females revealed a significant main effect for drug condition for females, but not for males [$F(2,57) = 4.40$, $p < .05$ and $[F(2,57) = 1.32$, n.s., respectively]. Tukey HSD *post-hoc* analyses indicated that female rats exposed to 12 mg nicotine/kg/day continued to weigh significantly less than did female rats administered saline. The main effects for sex [$F(1,108) = 890.84$, $p < .05$] and for drug condition [$F(2,108) = 3.28$, $p < .05$] continued during days 3 and 4 of nicotine or saline cessation. However, separate one-way ANOVAs for males and females did not reveal significant main effects for drug condition. There no longer was a significant main effect for drug on the last 2 days (days 6-7) of nicotine cessation [$F(2,108) = 2.72$, n.s.], but the main effect for sex remained [$F(1,108) = 3.28$, $p < .05$].

Food consumption. Analysis of food consumption collapsed within each of the 12 treatment groups across this phase indicated that there still was a significant main effect for sex with males consuming more food than did females [$F(1,108) = 289.21$, $p < .05$] (see Figure 5). There were no main effects for drug or stress conditions and there were no significant 2- or 3-way interactions. These results are listed in Table 10.

Water consumption. Analysis of water consumption collapsed within each of the 12 treatment groups across this phase indicated that there was a significant main effect for sex with males drinking more water than did females [$F(1,75) = 7.55$, $p < .05$] (see Figure 6). There no longer was a main effect for stress condition [$F(1,75) = 1.64$, n.s.] and there was no main effect for drug condition. There also were no significant 2- or 3-way interactions. These results are listed in Table 11. Several animals were not included in

this analysis because of missing data on day 1 of the cessation phase. Therefore, a separate 3-way ANOVA was conducted on mean water consumption during the last 3 days of this phase. This second analysis also was performed to insure that there were no significant differences in water consumption between animals that were assigned to stress and no-stress conditions. Results indicated that there was no main effect for stress on water consumption during the last 3 days of nicotine cessation (see Table 12 for results). This finding was important because it indicated that water consumption did not need to be used as a covariate variable when evaluating opioid consumption during the opioid self-administration phases of the experiment.

Opioid Initiation

Following nicotine or saline cessation, fentanyl solution was made available to all subjects to examine drug self-administration. On each day, animals had access to fentanyl solution alone [fentanyl only (FO)] or they had access to the opioid solution and tap water [choice (CH)]. FO days and CH days were cycled every 5 days with 1 CH day and 4 FO days. The first 3 days of initiation were CH days in order to examine initial preference (or choice) for the fentanyl solution and the last 5 days consisted of 1 CH day and 4 FO days. In order to make comparisons between male and female rats, fentanyl consumption was adjusted for body weight and fentanyl amounts were calculated for each animal for each day of fentanyl exposure. Fentanyl amounts are presented as milligrams (mg) of fentanyl per kilogram (kg) of body weight. Opioid initiation was evaluated during the 8 days (days 36-43) of initial exposure to the fentanyl solution. In addition to exposing subjects to the fentanyl solution, the stress manipulation (IM) occurred every day, 20 minutes prior to the

fentanyl access period.

Body weight, food and water consumption. Analysis of body weights collapsed within each of the 12 treatment groups across the 8 days of the initiation phase indicated that there was a significant main effect for sex with males weighing more than females [$F(1,108) = 904.55$, $p < .05$] (see Figure 4). There were no other significant main effects or interactions (see Table 13 for results). With respect to food and water consumption during the 18-hour time period spent without the drug, there was a main effect for sex for both dependent variables [$F(1,108) = 302.11$, $p < .05$ and [$F(1,108) = 35.09$, $p < .05$, respectively] with males eating and drinking more than did females. These results are listed in Tables 14 and 15.

Opioid Consumption. Figure 7 presents mean fentanyl consumption (mg/kg) by male and female rats during the opioid initiation phase. A 3-way ANOVA was conducted on mean fentanyl consumption during the initiation phase. As predicted, there was a main effect for sex during the initiation phase with females consuming almost twice as much fentanyl as did males [$F(1,108) = 41.15$, $p < .05$]. However, there were no main effects for stress (see Figure 8) or drug conditions (see Figures 9 and 10) and there were no significant 2- or 3-way interactions (see Table 16 for results). To determine any possible effects that nicotine exposure might have had on fentanyl initiation, drug consumption by animals exposed to both dosages of nicotine ($n = 80$) was compared with drug consumption by animals exposed to saline ($n = 20$) using a 3-way ANOVA with 2 levels of drug (i.e., 6 and 12 mg nicotine/kg/day) as one of the independent variables. Again, there was a main effect for sex [$F(1,112) = 34.35$, $p < .05$], but there were no main

effects for drug or stress conditions and there were no significant 2- or 3-way interactions (see Table 17 for results). Because of the gender difference in drug consumption (which was significant based on amount by weight and based on absolute amount), two-way ANOVAs were conducted separately for male and female fentanyl consumption with stress and drug as the independent variables. When conducted separately, there were no significant main effects or 2-way interactions among male or among female rats.

There also was a main effect for sex on fentanyl preference during the 4 CH days of initiation [$F(1,90) = 5.86$, $p < .05$] and a sex X stress X drug interaction [$F(2,90) = 3.52$, $p < .05$] (see Figure 11). Specifically, females had a greater preference for the fentanyl solution than did males. There were no other significant main effects or 2-way interactions (see Table 18 for results). These same effects held when nicotine groups were collapsed and fentanyl preference was compared among nicotine and saline animals. Specifically, there was a main effect for gender [$F(1,94) = 5.09$, $p < .05$], with females preferring the fentanyl solution more than did males, and a sex X stress X drug interaction [$F(2,90) = 6.21$, $p < .05$] (see Table 19 for results). Again, because of the sex difference in fentanyl preference, two-way ANOVAs were conducted separately for male and female fentanyl preference with stress and drug as the independent variables. When conducted separately, there were no significant main effects or 2-way interactions among male rats. Among females, however, there was a significant stress X drug interaction [$F(2,36) = 3.41$, $p < .05$]. Specifically, nicotine exposure increased preference for fentanyl when females were exposed to stress (45.09 ± 3.51) but decreased fentanyl preference by non-stressed subjects (33.65 ± 2.78) compared with fentanyl preference by stressed ($36.70 \pm$

3.90) and non-stressed (42.27 ± 4.17) females previously exposed to saline. Subsequent one-way ANOVAs were conducted separately for male and female stress and no-stress rats with drug condition as the independent variable. Results indicated that there were no main effects for fentanyl preference between nicotine groups within any of the stress and no-stress, male and female rats.

Opioid Maintenance

Fentanyl access continued in 5-day cycles as described for the opioid initiation phase of the experiment and ended with an additional CH day. This maintenance phase lasted 21 days for a total of 5 CH days and 16 FO days (i.e., 4 cycles plus an additional CH day; days 44-64).

Analysis of body weights collapsed within each of the 12 treatment groups across the entire maintenance phase indicated that there was a significant main effect for sex with males weighing more than did females [$F(1,106) = 824.71$, $p < .05$] (see Figure 4). There also was a main effect for stress with stressed animals weighing significantly less than did non-stressed animals [$F(1,106) = 19.48$, $p < .05$]. Separate one-way ANOVAs were conducted for males and females with stress condition as the independent variable. Results were significant for males [$F(1,59) = 15.83$, $p < .05$] and females [$F(1,57) = 4.47$, $p < .05$]. There was no main effect for drug condition and there were no significant 2- or 3-way interactions (see Table 20 for results). With respect to food and water consumption during the 18-hour time period spent without the drug, there was a main effect for sex for both dependent variables [$F(1,106) = 95.67$, $p < .05$ and [$F(1,106) = 35.09$, $p < .05$, respectively] with males consuming more food and water than did females.

There also was a significant main effect for stress [$F(1,106) = 13.56$, $p < .05$] with stressed animals consuming less food than did non-stressed animals. There were no other significant differences in food and water consumption.

Opioid Consumption. Figure 7 presents mean fentanyl consumption (mg/kg) by male and female rats during the maintenance phase of the experiment. A 3-way ANOVA was conducted on mean fentanyl consumption during the maintenance phase. Again, there was a main effect for sex during the maintenance phase with females consuming more fentanyl than did males [$F(1,106) = 64.97$, $p < .05$] and there were no main effects for stress (see Figure 8) or drug conditions (see Figures 9 and 10). There also were no significant 2- or 3-way interactions (see Table 21 for results). To determine any possible effects that nicotine exposure might have had on maintenance of fentanyl self-administration, drug consumption by animals exposed to both dosages of nicotine ($n = 78$) was compared with drug consumption by animals exposed to saline ($n = 20$) using a 3-way ANOVA with 2 levels of drug as one of the independent variables. Again, there was a main effect for sex [$F(1,110) = 51.30$, $p < .05$], but there were no main effects for drug or stress conditions and there were no significant 2- or 3-way interactions (see Table 22 for results). Because of the gender difference in drug consumption, two-way ANOVAs were conducted separately for male and female fentanyl consumption with stress and drug (3 levels) as the independent variables. When conducted separately, there were no significant main effects or 2-way interactions among female rats. However, there was a significant stress X drug interaction among male rats [$F(2,54) = 4.36$, $p < .05$]. Subsequent one-way ANOVAs were conducted to investigate the main effect for sex and this interaction.

Results revealed a significant main effect for nicotine exposure on fentanyl consumption among male stressed rats [$F(1,27) = 3.30$, $p = .05$]. Tukey-HSD test indicated that stressed male rats exposed to 6 mg nicotine/kg/day self-administered less fentanyl than did stressed male rats exposed to saline. In contrast, there was a marginal main effect for nicotine exposure on fentanyl consumption among non-stressed male rats in the opposite direction [$F(1,27) = 3.05$, $p = .06$]. Specifically, male non-stressed rats exposed to 6 mg nicotine/kg/day self-administered more fentanyl than did non-stress male rats exposed to 12 mg nicotine/kg/day or to saline.

In order to test the gateway hypothesis among females, one-way ANOVA was conducted for the non-stressed animals with prior nicotine exposure as the independent variable. Results indicated that prior nicotine exposure did not significantly increase fentanyl consumption in adulthood [$F(2,27) = 0.84$, n.s.].

There also was a main effect for sex on fentanyl preference during the 5 CH days of maintenance [$F(1,106) = 6.04$, $p < .05$] with females preferring the fentanyl solution more than did males (see Figure 12). Interestingly, the sex X stress X drug interaction revealed during initiation no longer was significant during maintenance [$F(2,106) = 1.83$, n.s.]. There also were no other significant main effects or 2-way interactions (see Table 23 for results). There was a main effect for gender when nicotine groups were collapsed and fentanyl preference was compared among nicotine and saline animals [$F(1,110) = 6.13$, $p < .05$]. Females preferred the fentanyl solution more than did males. There were no other significant effects (see Table 24 for results). Again, because of the gender difference in fentanyl preference, two-way ANOVAs were conducted separately for male

and female fentanyl preference with stress and drug as the independent variables. When conducted separately, there were no significant main effects or 2-way interactions among male rats. Among females, the significant stress X drug interaction revealed during initiation did not hold during maintenance. Subsequent one-way ANOVAs were conducted separately for male and female stress and no-stress rats with drug condition as the independent variable. Results indicated that there were no main effects for fentanyl preference between nicotine groups within any of the stress and no-stress, male and female rats.

Opioid Withdrawal Assessment

Figure 13 presents total withdrawal behaviors observed in response to naloxone injection after opioid self-administration. Following the opioid maintenance phase, subjects were exposed to two additional choice days in order to evaluate opioid withdrawal behaviors. Half of the subjects (i.e., 60 subjects; 5 from each experimental group) received naloxone injections following fentanyl access on day 65 of the experiment and the second group of subjects received naloxone injections following fentanyl access on day 66. Behavioral assessments of opioid withdrawal behaviors (i.e., wet-dog shakes, diarrhea, mouthing and teeth chattering, ptosis, excessive grooming, abnormal posture) were made by two independent observers. The two observers achieved an inter-rater reliability coefficient of +0.90 prior to the experiment. Observation scores across the six categories of withdrawal symptoms were added together from each observer to compute an overall composite withdrawal score for each subject. This sum was calculated because observations by each observer were made at different intervals within the observation

period. In addition, there were no significant differences in total withdrawal behaviors observed on day 1 compared to withdrawal behaviors on day 2 [$F(2,116) = 0.01$, n.s.], therefore all subject data were analyzed together. Total withdrawal scores ranged from 1 to 107 and were analyzed by three-way ANOVA using sex, stress, and nicotine exposure as the independent variables. There were no significant main effects, 2-way, or 3-way interactions. The results from this analysis are presented in Table 25.

Decreased body weight as a result of withdrawal-induced diarrhea following naloxone injection also is used as an index of opioid withdrawal. An initial 3-way ANOVA was conducted on pre-injection body weight scores to determine potential sex differences in body weight. The results indicated a significant main effect for stress [$F(1,106) = 46.67$, $p < .05$], sex [$F(1,106) = 964.66$, $p < .05$], and a stress X sex interaction [$F(1,106) = 8.59$, $p < .05$]. That is, females weighed less than did males and stressed rats weighed less than did non-stressed rats prior to naloxone injection. There was no main effect for drug or any other 2-way or 3-way interactions. Table 26 presents the results from this analysis. To examine the effects of opioid withdrawal on body weight loss, repeated-measures ANOVAs were conducted separately for males and females with drug, stress, and time as the independent variables. The results revealed a significant effect for time for both males and females [$F(1,54) = 18.18$, $p < .05$ and $F(1,52) = 38.13$, $p < .05$, respectively]. Specifically, male and female rats lost a significant amount of body weight during the 20 minutes following naloxone injection. Table 27 presents pre- and post-injection body weight scores for males and females. There were no significant drug X time, stress X time, or stress X drug X time interactions.

In addition to the 12 experimental groups, a drug and stress naive control group (n = 8; 4 males and 4 females) was used to compare no-stress, no fentanyl control conditions to fentanyl stress and no-stress conditions on withdrawal scores. Figure 14 presents mean withdrawal scores for each experimental group and the no-drug, no-stress control group. A one-way ANOVA that compared total withdrawal scores observed in opioid-exposed animals with withdrawal behaviors observed in unexposed animals indicated that subjects that had self-administered fentanyl displayed significantly greater withdrawal behaviors than did animals that did not have previous access to the fentanyl solution [F (1,124) = 5.85, $p < .05$]. Specifically, animals that had access to the fentanyl solution exhibited almost 3 times as many withdrawal behaviors compared with animals that did not have access to fentanyl solution (36.29 ± 2.44 behaviors versus 13.50 ± 2.67 behaviors). The withdrawal scores of animals exposed to fentanyl solution are comparable to withdrawal scores that have been reported in the literature (Klein et al., 1997; Shaham et al., 1992) and are suggestive of opioid dependence.

A regression analysis with withdrawal score as the dependent variable and fentanyl self-administration during the maintenance phase of the experiment as the predictor variable was significant [R = +0.44, F (1,116) =26.06, $p < .05$]. That is, greater fentanyl consumption (mg/kg) was associated with greater withdrawal during the maintenance phase of the experiment. However, opioid consumption during the initiation phase of the experiment was not a significant predictor of opioid withdrawal behaviors [R = +0.11, F (1,116) =1.35, n.s.]. Despite the finding that females self-administered higher amounts of fentanyl (mg/kg) than did males during the maintenance phase, there were no significant

differences between the two groups for withdrawal scores. In addition, stress and prior nicotine exposure did not predict withdrawal behaviors in response to opioid antagonism.

Opioid Abstinence

Following the last day of withdrawal assessment, the fentanyl solution no longer was available to the animals and subjects had access only to tap water and food. No stress manipulation occurred and body weight, food and water consumption were measured for five days (days 67-71). Body weights and food and water consumption were collapsed within each of the 12 treatment groups across the 5-day abstinence phase.

Body weight. Three-way ANOVA indicated that there was a significant main effect for sex with males weighing more than females [$F(1,106) = 1044.69$, $p < .05$] (see Figure 4). There also was a significant main effect for stress condition with previously-stressed rats weighing less than previously non-stressed rats [$F(1,106) = 6.10$, $p < .05$]. There also was a significant drug X stress condition interaction [$F(2,106) = 14.80$, $p < .05$]. There were no main effects for drug condition and no other significant 2-way interactions (these results are listed in Table 28). Separate two-way ANOVAs were conducted for males and females and revealed a significant main effect for stress condition among male rats but not among female rats [$F(1,54) = 4.89$, $p < .05$ and $F(2,52) = 1.35$, n.s., respectively]. That is, stressed male rats weighed significantly less than did non-stressed male rats. There also was a significant drug X stress interaction for both male and female rats [$F(2,54) = 10.30$, $p < .05$ and $F(2,52) = 5.15$, $p < .05$, respectively]. To further examine the significant effects of stress and nicotine exposure on body weight, one-way ANOVAs were conducted separately for male and female, stressed and non-

stressed rats. These analyses revealed significant main effects for drug conditions for all groups except non-stressed female rats. Tukey HSD *post-hoc* test indicated that non-stressed male rats previously exposed to 6 mg nicotine/kg/day weighed significantly less than did non-stressed male rats previously exposed to 12 mg nicotine/kg/day (420.64 ± 9.39 g versus 469.70 ± 12.70 g). In contrast, stressed male rats previously exposed to 6 mg nicotine/kg/day weighed significantly more than did stressed male rats previously exposed to either 12 mg nicotine/kg/day or saline (459.89 ± 10.90 g versus 410.53 ± 9.07 and 412.60 ± 6.95 g; Tukey HSD *post-hoc* test). Similarly, stressed female rats previously exposed to 6 mg nicotine/kg/day weighed significantly more than did stressed female rats previously exposed to 12 mg nicotine/kg/day (271.58 ± 6.73 g versus 247.43 ± 2.60; Tukey HSD *post-hoc* test).

Food consumption. Three-way ANOVA indicated that there was a significant main effect for sex with males consuming more food than did females [$F(1,106) = 234.68$, $p < .05$] (see Figure 5). Similar to the results with body weight, there also was a significant drug X stress condition interaction [$F(2,106) = 5.18$, $p < .05$]. However, there were no main effects for stress or drug condition, there were no other significant 2-way interactions, and there was no significant 3-way interaction (these results are presented in Table 29). Separate two-way ANOVAs conducted for males and females revealed a significant stress X drug condition interaction among male rats [$F(2,54) = 4.40$, $p < .05$] but not among female rats. There also were no main effects for stress or drug condition. To further examine the significant effects of stress and nicotine exposure on food consumption, one-way ANOVAs were conducted separately for male and female, stressed

and non-stressed rats. These analyses revealed a significant main effect for drug condition only for stressed female rats [$F(2,27) = 5.09$, $p < .05$]. Specifically, Tukey HSD *post-hoc* analysis revealed that stressed female rats previously exposed to 6 mg nicotine/kg/day ate significantly more food than did stressed females rats previously exposed to saline (24.02 ± 0.64 g versus 21.80 ± 0.46 g). When groups were analyzed across drug condition, one-way ANOVA revealed a significant effect for stress among male rats previously exposed to 6 mg nicotine/kg/day [$F(1,18) = 6.32$, $p < .05$]. Specifically, among male rats in this previous drug condition, stressed rats consumed less food (29.07 ± 0.74 g) than did non-stressed rats (24.02 ± 0.64 g).

Water consumption. Analysis of water consumption collapsed within each of the 12 treatment groups across this phase indicated that there was a significant main effect for sex with males drinking more water than did females [$F(1,106) = 10.74$, $p < .05$] (see Figure 6). There were no main effect for stress condition or drug condition and there were no significant 2- or 3-way interactions. These results are listed in Table 30.

Corticosterone

Figure 15 presents mean plasma corticosterone levels (ng/ml) in saline or nicotine (6 or 12 mg nicotine/kg/day) exposed male and female rats on the last day of the experiment following either 20 minutes of immobilization stress or no-stress. Corticosterone levels at the end of the experiment were analyzed with a three-way ANOVA using sex, stress, and nicotine exposure as the independent variables. As predicted, immobilization stress resulted in higher levels of plasma corticosterone compared with the no-stress condition [$F(1,106) = 376.10$, $p < .05$] and female rats had

higher levels of plasma corticosterone than did male rats [$F(1,106) = 88.49$, $p < .05$], regardless of stressor condition. There also was a main effect for nicotine exposure during adolescence on plasma corticosterone during adulthood [$F(2,106) = 4.78$, $p < .05$] with prior nicotine exposure resulting in higher amounts of corticosterone. There also was a significant nicotine history by stress interaction [$F(2,106) = 13.75$, $p < .05$] and there was a significant sex by stress by nicotine history interaction [$F(2,106) = 3.07$, $p = .05$]. These results are listed in Table 31. Subsequent one-way ANOVAs revealed that there were no differences in plasma corticosterone among the male and female stressed animals with varying nicotine history [$F(2,27) = 0.43$, n.s. and $F(2,27) = 27$, n.s., respectively]. However, among the no-stress groups, nicotine exposure resulted in increased plasma corticosterone levels following opioid consumption in male and female rats [$F(2,27) = 9.42$, $p < .05$ and $F(2,25) = 7.70$, $p < .05$, respectively]. Tukey HSD *post-hoc* analyses were conducted separately to examine the effects of adolescent nicotine exposure on corticosterone levels among male and female no-stress animals. These results indicated that previous exposure to the 12 mg nicotine/kg/day dose produced significant increases in plasma corticosterone levels and that previous exposure to the 6 mg nicotine/kg/day dose marginally increased plasma corticosterone levels for non-stressed male rats compared to prior saline exposure for non-stressed male rats. Among the non-stressed female rats, prior exposure to either 6 or 12 mg nicotine/kg/day produced significant increases in plasma corticosterone compared to prior saline exposure.

A regression analysis with plasma corticosterone values as the predictor variable and fentanyl consumption by all subjects during the initiation phase of the experiment as

the dependent variable revealed a significant correlation [R= +0.19, $\underline{F}$ (1, 116) = 4.46, $\underline{p}$ < .05]. This relationship did not hold during the maintenance phase of the experiment [R= 0.15, $\underline{F}$ (1, 116) = 2.82, n.s.]. In other words, greater plasma corticosterone levels were positively correlated with fentanyl SA during initiation but not during maintenance.

CONFIRMATION OF HYPOTHESES

The present experiment was designed to test two major hypotheses and six minor hypotheses. Major Hypothesis 1 addressed the gateway hypothesis. Major Hypothesis 2 addressed the relationship between stress and drug self-administration. Minor Hypothesis 1 addressed predicted differences between males and females in opioid self-administration. Minor Hypotheses 2 and 3 addressed predictions regarding opioid withdrawal. Minor Hypothesis 4 addressed stress, sex, and plasma corticosterone. Minor Hypotheses 5 and 6 addressed predictions regarding nicotine and body weight.

Major Hypotheses

Major Hypothesis 1: This hypothesis was only **partially confirmed**. Non-stressed male rats exposed to 6 mg nicotine/kg/day consumed greater amounts (mg/kg) of fentanyl than did saline controls. The non-stressed male rats exposed to 12 mg nicotine/kg/day were indistinguishable from controls with regard to subsequent fentanyl consumption. Nicotine exposure may have been related to subsequent fentanyl self-administration by non-stressed male rats in an inverted-U shaped function, but additional dosages are necessary to confirm the shape of this function. This effect of nicotine on opioid consumption did not occur among stressed male rats. In fact, exposure to immobilization stress attenuated or reversed the effect of adolescent exposure to 6 mg nicotine/kg/day on opioid consumption in adulthood. Non-stressed female rats exposed to 12 mg nicotine/kg/day consumed somewhat greater amounts (mg/kg) of fentanyl than did their saline and 6 mg nicotine/kg/day counterparts but this effect was not significant. This effect of nicotine on opioid consumption did not occur among stressed female rats. In

fact, exposure to immobilization stress attenuated any effects of adolescent exposure to nicotine (6 or 12 mg nicotine/kg/day) on opioid consumption in adulthood.

Major Hypothesis 2: This hypothesis was **not confirmed**. There was no effect of immobilization stress on opioid consumption by male or female rats.

Minor Hypotheses

Minor Hypothesis 1: The hypothesis that adult female rats would self-administer significantly greater amounts (mg/kg) of fentanyl than would adult male rats, regardless of previous nicotine exposure, was **confirmed**.

Minor Hypothesis 2: The finding that there were no sex differences in opioid withdrawal behaviors in response to opioid antagonism **disconfirmed** this hypothesis.

Minor Hypothesis 3: The hypothesis that male rats exposed to immobilization stress would exhibit a greater number of withdrawal behaviors in response to naloxone challenge than would male and female rats not exposed to stress and female rats exposed to stress was **disconfirmed**.

Minor Hypothesis 4: The hypothesis that rats exposed to immobilization stress would have higher levels of plasma corticosterone than would rats that were not exposed to immobilization stress was **confirmed**. Also, female rats had higher levels of plasma corticosterone than did male rats, confirming this hypothesis.

Minor Hypothesis 5: The hypothesis that rats exposed to nicotine would gain less weight than would rats exposed to saline and that this weight difference would be greater in female rats was **confirmed**.

Minor Hypothesis 6: The hypothesis that rats exposed to nicotine would gain

more weight during the nicotine abstinence phase than would rats exposed to saline was **confirmed**. The finding that this weight difference was greater in female rats also confirmed this hypothesis.

DISCUSSION

The problem that inspired the present experiment is, "Why do some adolescents who smoke cigarettes go on to later use illicit drugs?" Although a range of variables, including social, psychological, behavioral, and biological, may contribute to this phenomenon, the present experiment focused on a psychopharmacological variable that may influence illicit drug use in adulthood. More specifically, the present experiment was designed to evaluate whether exposure during adolescence to nicotine, the primary active pharmacologic agent of addiction in tobacco, increases the likelihood of consuming opiates in adulthood. In addition, the present experiment included males and females in order to determine whether or not gender differences exist in this possible "gateway." Further, this experiment manipulated stress in order to determine whether or not stress affects any relationship between nicotine exposure and subsequent opiate self-administration. Male and female rats were the subjects to allow careful control of all of the independent variables as well as to expose the subjects to drugs and conditions that would not be ethical in a human investigation. Overall, the results indicated that exposure to a moderate dosage of nicotine increased subsequent opiate self-administration only by non-stressed male rats. This finding suggests that a psychopharmacologic explanation for the gateway hypothesis may hold for certain males, and that other variables (e.g., other biologic mechanisms, social variables, cultural variables) may be needed to explain the progression from tobacco to other drug use by stressed individuals and by females.

The effects of nicotine administration on subsequent oral fentanyl self-administration with and without stress were evaluated in a 3 (0, 6 mg, or 12 mg

nicotine/kg/day) x 2 (male, female) x 2 (immobilization stress, no-stress) experimental design. Adolescent rats received saline or nicotine (6 mg or 12 mg/kg/day) by osmotic minipump for 24 hours/day for 19 days. Then, following a 7-day wash-out period, subjects had access to fentanyl-HCl solution in their home cages for 6 hours/day every day for four weeks. Throughout this fentanyl self-administration phase, subjects were exposed to either 20 minutes/day of immobilization stress or no-stress prior to opioid availability. The dependent variables were: fentanyl consumption, body weight, food and water consumption, and plasma corticosterone.

Nicotine exposure during adolescence differentially affected male and female rats. Non-stressed, male rats exposed to 6 mg nicotine/kg/day consumed more fentanyl (mg/kg) than did non-stressed, male rats exposed to saline or 12 mg nicotine/kg/day. In contrast, this relationship between nicotine exposure and opiate consumption did not occur among stressed, male rats. In fact, exposure to the immobilization stress prior to opioid availability attenuated or reversed the effect of adolescent nicotine exposure to 6 mg nicotine/kg/day on subsequent fentanyl self-administration. For female rats, exposure to nicotine did not significantly alter subsequent fentanyl self-administration.

The findings for the male, non-stressed rats partially support a psychopharmacologic basis for the gateway hypothesis in that exposure to 6 mg nicotine/kg/day during adolescence resulted in an increase in opioid consumption during adulthood. The fact that exposure to 12 mg nicotine/kg/day did not result in increased opiate self-administration in non-stressed males suggests either that there is a level of nicotine above which a psychopharmacologic mechanism for the gateway hypothesis does

not apply, or that the continuous nicotine administration paradigm creates a psychopharmacologic condition that does not reflect the self-administration of illicit substances by humans. Another possibility is that the dosages of nicotine used in the present experiment, that have proven to be effective and meaningful in adult rats, may be too high for adolescent rats. Future studies of adolescent exposure to nicotine should include more dosages, particularly more low dosages of nicotine. Inclusion of additional dosages is necessary to reveal the shape of the dose-effect function that may be an inverted-U. Additional nicotine dosages also are needed to determine whether the sex difference that occurred is, indeed, a lack of a nicotine exposure effect in females or a shift in the dose-response curve that was not revealed by the inclusion of only two nicotine dosages. In addition, future studies should include a nicotine self-administration paradigm that allows for a bolus administration of nicotine (i.e., peaks and troughs of nicotine levels) that may be a necessary psychopharmacologic condition for the gateway hypothesis with higher dosages of nicotine.

The findings for the stressed males and the findings for the females (stressed or not stressed) did not support a psychopharmacologic basis for the gateway hypothesis. Perhaps stress interfered with the effects of nicotine on subsequent fentanyl self-administration. If this interpretation is correct, then it suggests that a psychopharmacologic explanation for the gateway hypothesis does not apply to individuals under stress. To further explore this possibility would require follow-up experiments that manipulate stress in different ways, including environmental, psychological, physical, or biological stressors. The fact that the results for the females do not support a

psychopharmacological mechanism for the gateway hypothesis may reveal that this explanation does not account for any opiate self-administration in females, or that the nicotine dosages were too high in the present experiment. Follow-up studies should include lower dosages of nicotine in adolescent rats. It also is possible that female subjects were more sensitive to nicotine than were male subjects and that the self-administration of fentanyl for females reflected the descending limb of an inverted U-shaped function. This possibility is consistent with other reports that females are more sensitive to nicotine than are males (Bättig, 1981; Silverstein et al., 1980). The inclusion of lower dosages in follow-up studies would help delineate the shape of the function and would examine this possibility of a sex difference in sensitivity to nicotine with regard to the gateway hypothesis. An alternate explanation is that the females were less sensitive to the rewarding effects of the nicotine and, therefore, were less likely to self-administer fentanyl during adulthood. A recent report by Perkins (1996) suggests that nicotine may be less reinforcing for women compared with men. Future studies should include a nicotine self-administration paradigm in order to allow the examination of the reinforcing efficacy of nicotine in male versus female rats and how this reinforcement affects subsequent opioid self-administration. A third explanation for this lack of support for a psychopharmacologic mechanism in the gateway hypothesis for female rats is that they are less sensitive to the reinforcing effects of the fentanyl. Lex (1991) reported that women and men use and abuse different drugs of addiction. For example, women are more likely to abuse psychotherapeutic drugs such as sedatives, tranquilizers, or analgesics compared to men who are more likely to abuse alcohol, marijuana, or cocaine. Future studies should

provide choices of target drugs (e.g., amphetamines, benzodiazepines) in order to further examine gender differences in psychopharmacologic conditions in which use of one drug leads to the use of another drug.

Nicotine exposure during adolescence also affected other dependent measures. Nicotine exposure during adolescence increased plasma corticosterone levels in non-stressed, male and female rats. This effect of nicotine on plasma corticosterone was revealed in subjects that had been exposed to nicotine roughly two months earlier. Therefore, nicotine exposure during adolescence appeared to have a biochemical effect that lasted into young adulthood. This increase, however, did not occur among stressed rats. In addition, this finding was based on plasma corticosterone levels collected at only one time point. Exposure to nicotine during adolescence may have long-lasting biochemical effects that could be relevant to other behaviors (e.g., eating, hyperactivity, attention) and biological responses (e.g., hormones, neurochemicals), but this finding should remain tentative until replicated and confirmed in studies that include more treatment groups and more samples to analyze plasma corticosterone over the course of the study.

Nicotine exposure also decreased body weight gains and food consumption among male and female rats. Both of these effects were greater in female than in male rats. Nicotine cessation resulted in significant increases in body weight and food consumption and these effects were greater in females than in males. These findings replicate previous reports in Sprague-Dawley rats (Grunberg, 1992; Grunberg et al, 1985; Grunberg et al, 1984; Grunberg et al., 1988; Grunberg et al., 1987) and indicate that the effects of

nicotine on body weight and food consumption occur in adolescents as well as in adults.

Female rats consumed significantly more fentanyl (mg/kg) than did male rats, regardless of nicotine pre-exposure. In addition, female rats had a greater preference for the fentanyl solution than did the male rats. These sex differences in fentanyl self-administration are consistent with earlier studies using opioids, including fentanyl (Brown et al., 1995; Klein et al., 1997) and morphine (Alexander et al., 1978). These differences in drug self-administration and fentanyl preference may suggest a sex difference in sensitivity or tolerance to the fentanyl. It could be that female rats were more sensitive to the rewarding and addicting actions of fentanyl and, therefore, consumed and preferred the fentanyl solution more. Alternatively, it could be that female rats were less sensitive to the rewarding effects of fentanyl and, therefore, needed to consume more fentanyl to achieve a similar effect as experienced by the males. In this context, it is relevant that male and female rats did not display significant differences in the number of withdrawal behaviors observed following naloxone challenge. So, females consumed more fentanyl (mg/kg) than did males but displayed similar amounts if opioid withdrawal which suggests that females are less sensitive to fentanyl than are males. These findings also support the earlier explanation that the gateway may not have occurred for females because the fentanyl was less reinforcing for them compared with the males. It is noteworthy that a recent report by Klein and colleagues (1997) indicated that male rats displayed greater amounts of withdrawal behavior following naloxone challenge than did female rats despite lower amounts of fentanyl consumption. The dosage of fentanyl used in that study was similar to the dosage used in the present experiment. In order to directly examine these

different possibilities, male and female rats could be provided with different dosages of fentanyl and preference for each dosage could be evaluated to more fully reveal the underlying dose-effect curve. In addition, it would be valuable to measure blood and brain levels of fentanyl in males and females to determine if there are gender differences in fentanyl metabolism, distribution, elimination, and reabsorption. Moreover, additional behavioral (e.g., locomotion, acoustic startle response, pain perception) and biological (e.g., biochemical, physiological) dependent variables could be evaluated in response to fentanyl consumption in male and female rats. Considering possible sex differences in sensitivity to fentanyl, it is noteworthy that two female rats, not exposed to stress, died of apparent fentanyl overdose. These two rats also had been exposed to 12 mg nicotine/kg/day and died despite lower amounts of fentanyl consumed in comparison with the stressed females that also had been exposed to 12 mg nicotine/kg/day. These deaths may reflect a heightened sensitivity to the fentanyl in adult female subjects that had been exposed to nicotine during adolescence. If this interpretation is correct, then there may be a cross-sensitization between nicotine and fentanyl. If this cross-sensitization occurs, then it means that the effects of adolescent nicotine exposure must be long-lasting even after nicotine cessation. To further investigate these sex differences, it also would be valuable to manipulate sex hormones and to investigate stress and opiate consumption.

Separate from effects of nicotine and fentanyl, there also were effects of stress and sex in the present experiment. First, stress significantly increased plasma corticosterone in male and female rats. In addition, female rats had higher levels of plasma corticosterone. Both of these findings replicate previous reports (Brown & Grunberg, 1995; Kant et al.,

1983; Kant et al., 1987; Klein et al., 1997; Raygada et al., 1992; Shaham et al., 1993). Second, stress significantly decreased body weight and food consumption in male and female rats regardless of nicotine history. This finding is consistent with previous reports but extends them by using a repeated stressor and by evaluating food consumption over long periods of time (Greeno & Wing, 1994; Grunberg & Klein, 1995; Grunberg & Straub, 1992; Klein, Lapidus & Grunberg, 1995; Klein, Faraday, & Grunberg, 1996; Zylan & Brown, 1996).

Another finding was that opiate self-administration decreased food consumption for all animals. This finding is consistent with reports that different drugs (e.g., opiates, cocaine, amphetamines) affect eating behavior and that food availability affects drug self-administration (Carroll & Meisch, 1984; Grunberg, 1986; Kanarek & Marks-Kaufman, 1988; Levine & Morley, 1983; Marks-Kaufman, 1982; Marks-Kaufman & Liples, 1982).

An unexpected finding was that immobilization stress did not significantly alter fentanyl consumption in male and female rats. Specifically, it was hypothesized that exposure to stress would result in increased fentanyl self-administration by male rats and that stressor exposure would result in decreased fentanyl consumption by female rats. There are a few possible explanations for these unexpected results. First, it is possible that the dosage of fentanyl played a role in the effect of stress on drug consumption. Specifically, animals in this experiment were given access to a higher concentration of fentanyl-HCl (50 µl/ml) than were subjects in a previous investigation that examined the effects of immobilization stress on fentanyl (25 µl/ml) consumption by male rats (Shaham, 1993). The dosage of fentanyl in the present experiment was based on a similar dosage

used in earlier reports that examined the effects of predictable or unpredictable footshock on drug consumption in male rats (Klein et al., 1997; Shaham et al., 1993). Unpredictable footshock stress affected fentanyl consumption by male rats but not by female rats compared with no-stress conditions (Klein et al., 1997; Klein et al., 1993). The results of the present experiment suggest that different stressors (i.e., immobilization stress versus footshock stress) may differentially influence the relationship between stress and illicit drug consumption by altering the reinforcing value of the illicit drug. If this distinction is correct, then future studies should include different stressors and different dosages of opioids. In this context, it also is relevant that housing conditions can affect stress responses differently in male and female rats (Brown & Grunberg, 1995). Therefore, the failure to find an effect of immobilization stress in the females in the present experiment may be somewhat related to effects of the individual housing conditions used in the present study. Future studies should consider this possibility.

Another potential reason for this lack of an effect of stress on drug consumption is the age of the subjects in the present experiment. The age of the male subjects during the time of opioid availability (i.e., 68-96 days old) was similar to the ages of subjects (60-185 days) in other studies that have investigated the effects of stress on opioid consumption in male rats (Klein et al., 1997; Shaham et al., 1992; Shaham et al., 1993). However, females in the present experiment were much younger than were females included in the one published report on the effects of stress on opioid consumption (i.e., 68-96 days of age versus 105-185 days of age; Klein et al., 1997). Therefore, it would be valuable to study effects of stress on male and female subjects of different ages.

In addition, animals in the present experiment were exposed to other experimental conditions (e.g., minipump implant and explant) prior to the opioid phase of the experiment, whereas subjects in other studies were experimentally naive before the beginning of the experiment. Therefore, it is possible that the behavioral history (e.g., housing conditions, handling) of the animals during adolescence played an important role in influencing the effects of stress on fentanyl consumption. Other studies have revealed that housing and rearing conditions can influence opioid consumption in rats (Alexander et al., 1981; Alexander et al., 1978; Bardo, Robinet, & Hammer, 1997; Bozarth et al., 1989; Brown et al., 1995; Marks-Kaufman & Lewis, 1984; Pilcher & Jones, 1981; Schenk, Britt, Atalay, & Charleson, 1982; Schenk, Ellison, Hunt, & Amit, 1985; Zimmerberg & Brett, 1992). Specifically, rats under 8 weeks of age that are raised in isolation (i.e., individual housing conditions) consume more opioids and are less sensitive to the effects of the opioids (i.e., less severe withdrawal symptoms following naloxone injections, decreased opioid receptor binding) compared with animals raised in groups (Alexander et al., 1981; Alexander et al., 1978; Marks-Kaufman & Lewis, 1984; Schenk et al., 1982; Schenk et al., 1985). Therefore, it is possible that isolated housing conditions played a more important role in influencing fentanyl consumption than did restraint stress in the present experiment. In addition, differential housing conditions during adolescence may have influenced the effects of nicotine on illicit drug consumption during adulthood. Future studies should include differential housing conditions (e.g., isolated, grouped, crowded) during development and during nicotine exposure to clarify the role that nicotine, stress, or both may play on opioid consumption during adulthood. In addition, the present experiment

only examined the effects of stress on drug consumption in adulthood after adolescence and nicotine exposure. Future studies should evaluate the interactive effects of stress and nicotine exposure during adolescence on subsequent drug self-administration during adulthood.

There are several additions and changes that would be worthwhile in follow-up experiments based on the present findings. First, it would be valuable to include two additional experimental groups to further investigate the effects of stress on drug consumption: one group that is exposed to stress during opioid initiation but not during opioid maintenance; and one group that is not exposed to stress during opioid initiation but is exposed to stress during opioid maintenance. These two groups would help clarify the role that stress may play in the initiation and in the maintenance of drug-taking behavior. In the present experiment, it is possible that the effects of stress on opioid consumption were not evident because stress does not affect the initiation of drug-taking behavior. The present experiment examined drug self-administration for a relatively short period of time compared with other studies that have reported an effect of stress on opioid consumption (e.g., Klein et al.,1997; Shaham et al., 1992; Shaham et al., 1993). For example, Klein and colleagues (1997) reported that sex differences in fentanyl consumption manifested within the first 3 days of opioid availability. However, the effects of predictable versus unpredictable stress on fentanyl consumption did not occur until at least 20 days of opioid access. Further, reports by Shaham and colleagues (Shaham, 1992; Shaham et al., 1992; Shaham et al., 1993) indicated that effects of stress (immobilization, footshock) on opioid consumption in male rats were found over a 50-day drug availability

period. In addition, these other experiments used water deprivation, forced consumption, and gradual increases in drug concentration to induce drug consumption. By initially providing a choice of fentanyl and water, the present experiment was designed to investigate the effects of nicotine exposure during adolescence on opioid consumption in adulthood in a way that more closely resembled the human condition. Fentanyl-only days were included in this study because previous investigations have used this paradigm of drug-consumption induction. With respect to the gateway hypothesis, however, children and adults alike have a choice between taking or not taking drugs that are available on the street. In addition, there is little choice as to the concentration of a particular drug that a person may receive off the street. If this is the case, then another modification to the present paradigm would be the continuous availability of water and opioids in the home cage.

Another addition to the present experiment would be the cessation of the stressor and the reinstatement of the stressor in order to investigate the effects of stress on relapse of drug-taking behavior. Stressors may reinstate drug-seeking behavior because they activate neural pathways, such as the mesolimbic dopaminergic system, that are similar to those activated by the rewarding drugs themselves (Koob & Bloom, 1988; Shaham & Stewart, 1995, 1996; Stewart, 1984; Stewart & Vezina, 1988; Wise & Bozarth, 1987). Another addition to the present paradigm would be the assessment of other biochemicals, including dopamine and its metabolites, in order to better understand their role in the gateway hypothesis. Future studies also should examine the role of opioid receptors in mediating any of these effects.

Another addition to the present experiment is the inclusion of: (1) a group of animals that are continuously provided with nicotine during adulthood to evaluate the effects of nicotine exposure from adolescence through adulthood on opioid consumption; (2) a group of animals that are not exposed to nicotine until adulthood to examine adult nicotine exposure on opioid consumption; and (3) a group of animals that are given nicotine during adolescence and adulthood and then are given saline to examine the effects of nicotine cessation on opioid consumption. These groups may be necessary to determine whether psychopharmacologic mechanisms are involved in the gateway hypothesis. Females and stressed subjects might need nicotine in the body with opiates to reveal the hypothesized effects. For example, nicotine exposure may prime the individual (biologically or psychologically) to some effects of opiates that influence opiate self-administration. The present paradigm also could be used to expose adolescent rats to alcohol, with and without nicotine, to evaluate the potential influence of this other purported gateway drug. Additional target drugs such as inhalants, cocaine, heroin, and methamphetamine could be added to the protocol to investigate potential differences in the role that adolescent nicotine exposure may play in subsequent drug self-administration.

Future studies also could examine genetic factors as they influence the role that nicotine might play in the gateway hypothesis. For example, rats that are more or less sensitive to the effects of nicotine could be used in the present paradigm to help determine genetic contributions in the psychopharmacological effects of nicotine exposure during adolescence on subsequent drug consumption. Future studies also could use this model to examine potential behavioral (e.g., pain perception, attention, arousal), neurobiological

(e.g., opioid receptors, serotonergic pathways, corticosterone), and non-pharmacological (e.g., environment) mechanisms that might underlie the role that nicotine exposure during adolescence may have in increasing the likelihood of subsequent drug self-administration.

If the present findings generalize to humans, then they suggest that, in boys, nicotine exposure (e.g., cigarette smoking) during adolescence could result in increased opioid use in adulthood. In addition, the present findings suggest that a psychopharmacologic effect of nicotine on subsequent drug use may hold true for light smokers but not heavy smokers during adolescence. If this extrapolation is true, then low-nicotine yield cigarettes and other tobacco products may be particularly dangerous for adolescents. These results are alarming given the continued increase in tobacco use by adolescents (USDHHS, 1994). Another potentially dangerous issue is the availability of over-the-counter nicotine replacement therapies that are available in a range of doses. The sale of these products is not regulated and if the psychopharmacologic actions of nicotine do, in fact, lead to subsequent drug abuse, then these products may become another gateway mechanism. In addition to the gateway hypothesis, the present experiment reported that exposure to stress and prior exposure to nicotine decreased opioid consumption. If this finding with rats is true for humans, then it is possible that nicotine administration, via transdermal nicotine patch, in addicted individuals actually might diminish the reported effects of stress on increased drug consumption.

In summary, the present experiment examined a psychopharmacologic mechanism in the gateway hypothesis by evaluating whether exposure during adolescence to nicotine increased the likelihood of consuming opiates in adulthood in an animal paradigm. In

addition, the present experiment examined: whether or not gender differences exist in this possible "gateway" and whether or not stress affects any relationship between nicotine exposure and subsequent opiate self-administration. The results suggest that nicotine exposure during adolescence may have some biological effect that increases the likelihood of subsequent drug abuse for non-stressed males. The results suggest that a psychopharmacologic explanation for the gateway hypothesis may not hold for stressed males or for females. However, it is important to repeat the study with additional dosages of nicotine and with nicotine exposure continuing during opioid availability to determine definitively whether the hypothesized nicotine exposure mechanism should be rejected for females and stressed individuals. If the findings of the present experiment generalize to humans, any nicotine exposure (e.g., cigarettes, cigars, chewing tobacco, nicotine gum) might predispose some boys to abuse harder drugs. The present findings also suggest that other variables (e.g., social, psychological, cultural, other biological) deserve additional research attention. Finally, the present experiment provides a potential animal model to help further investigate the psychopharmacologic effect of adolescent nicotine exposure on other drug abuse (e.g., cocaine, amphetamines) and other appetitive behaviors in adulthood.

TABLES

Table 1. Experimental design.

Sex (2)	X Drug (3)	X Stress (2)
Male (n=60)	0 mg/kg/day (n=20)	Stress (n=10)
		No-stress (n=10)
	6 mg nic/kg/day (n=20)	Stress (n=10)
		No-stress (n=10)
	12 mg nic/kg/day (n=20)	Stress (n=10)
		No-stress (n=10)
Female (n=60)	0 mg/kg/day (n=20)	Stress (n=10)
		No-stress (n=10)
	6 mg nic/kg/day (n=20)	Stress (n=10)
		No-stress (n=10)
	12 mg nic/kg/day (n=20)	Stress (n=10)
		No-stress (n=10)

I← Fentanyl SA →I

Total N = 120

Table 2. Timeline of the experiment and associated ages of subjects.

EXPERIMENT PHASE	EXPERIMENT DAYS	AGE OF SUBJECTS
Gentling (3 days)	1-3	33-35 days
Baseline (5 days)	4-8	36-40 days
Nicotine/Saline Minipump Implant	9	41 days
Nicotine/Saline Exposure (19 days)	10-28	42-60 days
Nicotine/Saline Minipump Explant	28	60 days
Nicotine/Saline Abstinence (7 days)	29-35	61-67 days
Opioid Initiation (8 days)	36-43	68-75 days
Opioid Maintenance (21 days)	44-64	76-96 days
Naloxone Challenge (2 days)	65-66	97-98 days
Opioid Abstinence (5 days)	67-71	99-103 days
Corticosterone Measurement	72	104 days

Table 3. Results for three-way ANOVA on mean body weight during the baseline phase of the experiment (5 days) by sex, drug (0, 6, or 12 mg nicotine/kg/day), and stress condition.

Analysis	F (d.f.)	*p* Value
Sex	F(1,108)= 115.09	$p < .05$[1]
Drug	F(2,108)= 0.03	n.s.
Stress	F(1,108)= 0.04	n.s.
Drug X Sex	F(2,108)= 0.02	n.s.
Drug X Stress	F(2,108)= 0.02	n.s.
Sex X Stress	F(1,108)= 0.002	n.s.
Sex X Stress X Drug	F(2,108)= 0.02	n.s.

[1]Males > Females

Table 4. Results for three-way ANOVA on mean food consumption during the baseline phase of the experiment (5 days) by sex, drug (0, 6, or 12 mg nicotine/kg/day), and stress condition.

Analysis	F (d.f.)	*p* Value
Sex	F(1,108)= 33.83	$p < .05$[1]
Drug	F(2,108)= 0.35	n.s.
Stress	F(1,108)= 0.61	n.s.
Drug X Sex	F(2,108)= 0.12	n.s.
Drug X Stress	F(2,108)= 0.68	n.s.
Sex X Stress	F(1,108)= 1.06	n.s.
Sex X Stress X Drug	F(2,108)= 0.80	n.s.

[1]Males > Females

Table 5. Results for three-way ANOVA on mean water consumption during the baseline phase of the experiment (5 days) by sex, drug (0, 6, or 12 mg nicotine/kg/day), and stress condition.

Analysis	F (d.f.)	*p* Value
Sex	F(1,108)= 0.91	n.s.
Drug	F(2,108)= 0.27	n.s.
Stress	F(1,108)= 4.25	$p < .05$[1]
Drug X Sex	F(2,108)= 1.69	n.s.
Drug X Stress	F(2,108)= 0.18	n.s.
Sex X Stress	F(1,108)= 3.49	n.s.
Sex X Stress X Drug	F(2,108)= 0.09	n.s.

[1]Stress > No-Stress

Table 6. Results for three-way ANOVA on mean body weight during nicotine or saline exposure (19 days) by sex, drug (0, 6, or 12 mg nicotine/kg/day), and stress condition.

Analysis	F (d.f.)	*p* Value
Sex	F(1,108)= 542.76	$p < .05$[1]
Drug	F(2,108)= 9.35	$p < .05$[2]
Stress	F(1,108)= 0.60	n.s.
Drug X Sex	F(2,108)= 0.05	n.s.
Drug X Stress	F(2,108)= 0.09	n.s.
Sex X Stress	F(1,108)= 0.18	n.s.
Sex X Stress X Drug	F(2,108)= 0.70	n.s.

[1]Males > Females
[2]Nicotine < Saline

Table 7. Results for three-way ANOVA on mean food consumption during nicotine or saline exposure (19 days) by sex, drug (0, 6, or 12 mg nicotine/kg/day), and stress condition.

Analysis	F (d.f.)	*p* Value
Sex	F(1,108)= 366.53	$p < .05$[1]
Drug	F(2,108)= 18.07	$p < .05$[2]
Stress	F(1,108)= 2.30	n.s.
Drug X Sex	F(2,108)= 0.36	n.s.
Drug X Stress	F(2,108)= 0.10	n.s.
Sex X Stress	F(1,108)= 0.09	n.s.
Sex X Stress X Drug	F(2,108)= 0.82	n.s.

[1]Males > Females
[2]Nicotine < Saline

Table 8. Results for three-way ANOVA on mean water consumption during nicotine or saline exposure (19 days) by sex, drug (0, 6, or 12 mg nicotine/kg/day), and stress condition.

Analysis	F (d.f.)	*p* Value
Sex	F(1,108)= 32.84	$p < .05$[1]
Drug	F(2,108)= 0.71	n.s.
Stress	F(1,108)= 9.62	$p < .05$[2]
Drug X Sex	F(2,108)= 1.92	n.s.
Drug X Stress	F(2,108)= 0.49	n.s.
Sex X Stress	F(1,108)= 0.96	n.s.
Sex X Stress X Drug	F(2,108)= 0.89	n.s.

[1]Males > Females
[2]Stress > No-Stress

Table 9. Results for three-way ANOVA on mean body weight during nicotine or saline cessation (6 days) by sex, drug (0, 6, or 12 mg nicotine/kg/day), and stress condition.

Analysis	F (d.f.)	*p* Value
Sex	F(1,108)= 932.20	$p < .05$[1]
Drug	F(2,108)= 3.52	$p < .05$[2]
Stress	F(1,108)= 1.26	n.s.
Drug X Sex	F(2,108)= 0.002	n.s.
Drug X Stress	F(2,108)= 0.08	n.s.
Sex X Stress	F(1,108)= 0.31	n.s.
Sex X Stress X Drug	F(2,108)= 1.43	n.s.

[1]Males > Females
[2]Nicotine < Saline

Table 10. Results for three-way ANOVA on mean food consumption during nicotine or saline cessation (6 days) by sex, drug (0, 6, or 12 mg nicotine/kg/day), and stress condition.

Analysis	F (d.f.)	*p* Value
Sex	F(1,108)= 289.21	$p < .05$[1]
Drug	F(2,108)= 1.22	n.s.
Stress	F(1,108)= 2.77	n.s.
Drug X Sex	F(2,108)= 0.12	n.s.
Drug X Stress	F(2,108)= 0.14	n.s.
Sex X Stress	F(1,108)= 0.01	n.s.
Sex X Stress X Drug	F(2,108)= 1.97	n.s.

[1]Males > Females

Table 11. Results for three-way ANOVA on mean water consumption during nicotine or saline cessation (6 days) by sex, drug (0, 6, or 12 mg nicotine/kg/day), and stress condition.

Analysis	F (d.f.)	*p* Value
Sex	F(1,75)= 7.55	$p < .05$[1]
Drug	F(2,75)= 0.75	n.s.
Stress	F(1,75)= 1.64	n.s.
Drug X Sex	F(2,75)= 0.29	n.s.
Drug X Stress	F(2,75)= 0.52	n.s.
Sex X Stress	F(1,75)= 1.03	n.s.
Sex X Stress X Drug	F(2,75)= 2.27	n.s.

[1]Males > Females

Table 12. Results for three-way ANOVA on mean water consumption during the last 3 days of nicotine or saline cessation by sex, drug (0, 6, or 12 mg nicotine/kg/day), and stress condition.

Analysis	F (d.f.)	*p* Value
Sex	F(1,105)= 5.47	$p < .05$[1]
Drug	F(2,105)= 0.54	n.s.
Stress	F(1,105)= 3.67	n.s.
Drug X Sex	F(2,105)= 0.40	n.s.
Drug X Stress	F(2,105)= 0.12	n.s.
Sex X Stress	F(1,105)= 0.20	n.s.
Sex X Stress X Drug	F(2,105)= 0.43	n.s.

[1]Males > Females

Table 13. Results for three-way ANOVA on mean body weight during opioid initiation (8 days) by sex, drug (0, 6, or 12 mg nicotine/kg/day), and stress condition.

Analysis	F (d.f.)	*p* Value
Sex	F(1,108)= 904.66	$p < .05$[1]
Drug	F(2,108)= 2.39	n.s.
Stress	F(1,108)= 0.32	n.s.
Drug X Sex	F(2,108)= 0.001	n.s.
Drug X Stress	F(2,108)= 0.31	n.s.
Sex X Stress	F(1,108)= 0.02	n.s.
Sex X Stress X Drug	F(2,108)=1.70	n.s.

[1]Males > Females

Table 14. Results for three-way ANOVA on mean food consumption during 4 choice days of opioid initiation by sex, drug (0, 6, or 12 mg nicotine/kg/day), and stress condition.

Analysis	F (d.f.)	*p* Value
Sex	F(1,108)= 302.11	$p < .05$[1]
Drug	F(2,108)= 0.03	n.s.
Stress	F(1,108)= 2.60	n.s.
Drug X Sex	F(2,108)= 0.36	n.s.
Drug X Stress	F(2,108)= 0.45	n.s.
Sex X Stress	F(1,108)= 1.39	n.s.
Sex X Stress X Drug	F(2,108)= 0.86	n.s.

[1]Males > Females

Table 15. Results for three-way ANOVA on mean water consumption during 4 choice days of opioid initiation by sex, drug (0, 6, or 12 mg nicotine/kg/day), and stress condition.

Analysis	F (d.f.)	*p* Value
Sex	F(1,108)= 35.09	$p < .05$[1]
Drug	F(2,108)= 0.90	n.s.
Stress	F(1,108)= 1.56	n.s.
Drug X Sex	F(2,108)= 0.23	n.s.
Drug X Stress	F(2,108)= 0.21	n.s.
Sex X Stress	F(1,108)= 1.73	n.s.
Sex X Stress X Drug	F(2,108)= 1.01	n.s.

[1]Males > Females

Table 16. Results for three-way ANOVA on mean fentanyl consumption (mg/kg) during opioid initiation (8 days) by sex, drug (0, 6, or 12 mg nicotine/kg/day), and stress condition.

Analysis	F (d.f.)	*p* Value
Sex	F(1,108)= 41.15	$p < .05$[1]
Drug	F(2,108)= 0.45	n.s.
Stress	F(1,108)= 1.71	n.s.
Drug X Sex	F(2,108)= 0.41	n.s.
Drug X Stress	F(2,108)= 0.16	n.s.
Sex X Stress	F(1,108)= 2.51	n.s.
Sex X Stress X Drug	F(2,108)= 0.04	n.s.

[1]Males < Females

Table 17. Results for three-way ANOVA on mean fentanyl consumption (mg/kg) during opioid initiation (8 days) by sex, nicotine history (saline or nicotine), and stress condition.

Analysis	F (d.f.)	*p* Value
Sex	F(1,94)= 34.35	$p < .05$[1]
Nicotine History	F(1,94)= 0.94	n.s.
Stress	F(1,94)= 1.14	n.s.
Nicotine History X Sex	F(1,94)= 0.79	n.s.
Nicotine History X Stress	F(1,94)= 0.32	n.s.
Sex X Stress	F(1,94)= 2.04	n.s.
Sex X Stress X Drug	F(1,94)= 0.08	n.s.

[1]Males < Females

Table 18. Results for three-way ANOVA on mean fentanyl preference (percent) during 4 choice days of opioid initiation by sex, drug (0, 6, or 12 mg nicotine/kg/day), and stress condition.

Analysis	F (d.f.)	*p* Value
Sex	F(1,90)= 5.86	$p < .05$[1]
Drug	F(2,90)= 0.17	n.s.
Stress	F(1,90)= 1.05	n.s.
Drug X Sex	F(2,90)= 0.16	n.s.
Drug X Stress	F(2,90)= 1.01	n.s.
Sex X Stress	F(1,90)= 2.32	n.s.
Sex X Stress X Drug	F(2,90)= 3.52	$p < .05$[2]

[1]Males < Females
[2]Stress females with nicotine history > No-stress females with saline history > Stress females with saline history > No-stress females with nicotine history

Table 19. Results for three-way ANOVA on mean fentanyl preference (percent) during 4 choice days of opioid initiation by sex, nicotine history (saline or nicotine), and stress condition.

Analysis	F (d.f.)	*p* Value
Sex	F(1,112)= 5.09	$p < .05$[1]
Nicotine History	F(1,112)= 0.28	n.s.
Stress	F(1,112)= 0.37	n.s.
Nicotine History X Sex	F(1,112)= 0.23	n.s.
Nicotine History X Stress	F(1,112)= 1.24	n.s.
Sex X Stress	F(1,112)= 0.41	n.s.
Sex X Stress X Drug	F(1,112)= 6.21	$p < .05$[2]

[1]Males < Females
[2]Stress females with nicotine history > No-stress females with saline history > Stress females with saline history > No-stress females with nicotine history

Table 20. Results for three-way ANOVA on mean body weight during opioid maintenance (21 days) by sex, drug (0, 6, or 12 mg nicotine/kg/day), and stress condition.

Analysis	F (d.f.)	*p* Value
Sex	F(1,106)= 824.71	$p < .05$[1]
Drug	F(2,106)= 1.06	n.s.
Stress	F(1,106)= 19.48	$p < .05$[2]
Drug X Sex	F(2,106)= 0.06	n.s.
Drug X Stress	F(2,106)= 0.37	n.s.
Sex X Stress	F(1,106)= 3.74	n.s.
Sex X Stress X Drug	F(2,106)= 2.30	n.s.

[1]Males > Females
[2]Stress < No-Stress

Table 21. Results for three-way ANOVA on mean fentanyl consumption (mg/kg) during opioid maintenance (21 days) by sex, drug (0, 6, or 12 mg nicotine/kg/day), and stress condition.

Analysis	F (d.f.)	*p* Value
Sex	F(1,106)= 64.97	$p < .05$[1]
Drug	F(2,106)= 0.01	n.s.
Stress	F(1,106)= 2.11	n.s.
Drug X Sex	F(2,106)= 1.84	n.s.
Drug X Stress	F(2,106)= 1.60	n.s.
Sex X Stress	F(1,106)= 0.94	n.s.
Sex X Stress X Drug	F(2,106)= 0.89	n.s.

[1]Males < Females

Table 22. Results for three-way ANOVA on mean fentanyl consumption (mg/kg) during opioid maintenance by sex, nicotine history (saline or nicotine), and stress condition.

Analysis	F (d.f.)	*p* Value
Sex	F(1,110)= 51.30	$p < .05$[1]
Nicotine History	F(1,110)= 0.01	n.s.
Stress	F(1,110)= 0.75	n.s.
Nicotine History X Sex	F(1,110)= 1.47	n.s.
Nicotine History X Stress	F(1,110)= 1.98	n.s.
Sex X Stress	F(1,110)= 0.91	n.s.
Sex X Stress X Drug	F(1,110)= 0.05	n.s.

[1]Males < Females

Table 23. Results for three-way ANOVA on mean fentanyl preference (percent) during 5 choice days of opioid maintenance by sex, drug (0, 6, or 12 mg nicotine/kg/day), and stress condition.

Analysis	F (d.f.)	*p* Value
Sex	F(1,106)= 6.04	$p < .05$[1]
Drug	F(2,106)= 1.74	n.s.
Stress	F(1,106)= 2.31	n.s.
Drug X Sex	F(2,106)= 0.05	n.s.
Drug X Stress	F(2,106)= 0.05	n.s.
Sex X Stress	F(1,106)= 0.03	n.s.
Sex X Stress X Drug	F(2,106)= 1.83	n.s.

[1]Males < Females

Table 24. Results for three-way ANOVA on mean fentanyl preference (percent) during 5 choice days of opioid maintenance by sex, nicotine history (saline or nicotine), and stress condition.

Analysis	F (d.f.)	*p* Value
Sex	F(1,110)= 6.13	$p < .05$[1]
Nicotine History	F(1,110)= 3.07	n.s.
Stress	F(1,110)= 2.42	n.s.
Nicotine History X Sex	F(1,110)= 0.63	n.s.
Nicotine History X Stress	F(1,110)= 0.04	n.s.
Sex X Stress	F(1,110)= 0.41	n.s.
Sex X Stress X Drug	F(1,110)= 3.60	n.s.

[1]Males < Females

Table 25. Results for three-way ANOVA on total opioid withdrawal scores following naloxone injection (1.5 mg/kg) by sex, drug (0, 6, or 12 mg nicotine/kg/day), and stress condition.

Analysis	F (d.f.)	*p* Value
Sex	F(1,106)= 2.43	n.s.
Drug	F(2,106)= 0.58	n.s.
Stress	F(1,106)= 0.88	n.s.
Drug X Sex	F(2,106)= 0.94	n.s.
Drug X Stress	F(2,106)= 0.21	n.s.
Sex X Stress	F(1,106)= 0.70	n.s.
Sex X Stress X Drug	F(2,106)= 1.40	n.s.

Table 26. Results for three-way ANOVA on pre-naloxone injection (1.5 mg/kg) body weight by sex, drug (0, 6, or 12 mg nicotine/kg/day), and stress condition.

Analysis	F (d.f.)	*p* Value
Sex	F(1,106)= 964.66	$p < .05$[1]
Drug	F(2,106)= 0.66	n.s.
Stress	F(1,106)= 46.67	$p < .05$[2]
Drug X Sex	F(2,106)= 0.09	n.s.
Drug X Stress	F(2,106)= 0.24	n.s.
Sex X Stress	F(1,106)= 8.59	$p < .05$[3]
Sex X Stress X Drug	F(2,106)= 2.13	n.s.

[1]Males > Females
[2]Stress < No-Stress
[3]Stress males < Stress females < No-stress females < No-stress males

Table 27. Male and female pre-naloxone injection (1.5 mg/kg) and post-injection body weights (means and standard errors).

Group	Pre-injection Body Weight (g) ± SEM	Post-injection Body Weight (g) ± SEM
Males	430.02 ± 5.56	427.87 ± 5.63
Females	265.10 ± 3.00	261.48 ± 2.83

Table 28. Results for three-way ANOVA on mean body weight during opioid abstinence (5 days) by sex, drug (0, 6, or 12 mg nicotine/kg/day), and stress condition.

Analysis	F (d.f.)	*p* Value
Sex	F(1,106)= 1044.69	$p < .05$[1]
Drug	F(2,106)= 0.21	n.s.
Stress	F(1,106)= 6.10	$p < .05$[2]
Drug X Sex	F(2,106)= 1.16	n.s.
Drug X Stress	F(2,106)= 14.80	$p < .05$[3]
Sex X Stress	F(1,106)= 2.01	n.s.
Sex X Stress X Drug	F(2,106)= 3.27	$p < .05$[4]

[1]Males > Females

[2]Stress < No-Stress

[3,4]No-stress males with 6 mg nic/kg/day history < No-stress males with 12 mg nic/kg/day history; Stress males with 6 mg nic/kg/day history > Stress males with 12 mg nic/kg/day history and Stress males with saline history; Stress females with 6 mg nic/kg/day history > Stress females with 12 mg nic/kg/day history

Table 29. Results for three-way ANOVA on mean food consumption during opioid abstinence (5 days) by sex, drug (0, 6, or 12 mg nicotine/kg/day), and stress condition.

Analysis	F (d.f.)	*p* Value
Sex	F(1,106)= 234.68	$p < .05$[1]
Drug	F(2,106)= 0.93	n.s.
Stress	F(1,106)= 0.75	n.s.
Drug X Sex	F(2,106)= 0.79	n.s.
Drug X Stress	F(2,106)= 5.18	$p < .05$[2]
Sex X Stress	F(1,106)= 0.000	n.s.
Sex X Stress X Drug	F(2,106)= 0.59	n.s.

[1]Males > Females
[2]Stress females with 6 mg nic/kg/day history > Stress females with saline history

Table 30. Results for three-way ANOVA on mean water consumption during opioid abstinence (5 days) by sex, drug (0, 6, or 12 mg nicotine/kg/day), and stress condition.

Analysis	F (d.f.)	*p* Value
Sex	F(1,106)= 10.74	$p < .05$[1]
Drug	F(2,106)= 0.46	n.s.
Stress	F(1,106)= 0.48	n.s.
Drug X Sex	F(2,106)= 1.61	n.s.
Drug X Stress	F(2,106)= 0.90	n.s.
Sex X Stress	F(1,106)= 2.14	n.s.
Sex X Stress X Drug	F(2,106)= 0.75	n.s.

[1]Males > Females

Table 31. Results for three-way ANOVA on corticosterone levels (ng/ml) by sex, drug (0, 6, or 12 mg nicotine/kg/day), and stress condition.

Analysis	F (d.f.)	*p* Value
Sex	F(1,106)= 88.49	$p < .05$[1]
Drug	F(2,106)= 4.78	$p < .05$[2]
Stress	F(1,106)= 376.10	$p < .05$[3]
Drug X Sex	F(2,106)= 0.62	n.s.
Drug X Stress	F(2,106)= 13.75	$p < .05$[5]
Sex X Stress	F(1,106)= 3.59	$p = .06$[6]
Sex X Stress X Drug	F(2,106)= 3.07	$p = .05$[7]

[1]Males > Females
[2]Nicotine History > Saline
[3]Stress > No-Stress
[4,5,6,7]Stress males with saline or 6 mg nic/kg/day history or 12 mg nic/kg/day history = Stress females with saline or 6 mg nic/kg/day history or 12 mg nic/kg/day history > No-stress females with 12 mg nic/kg/day history > No-stress males with 12 mg nic/kg/day history > No-stress females with 6 mg nic/kg/day history >No-stress males with 6 mg nic/kg/day history > No-stress females with saline history > No-stress males with saline history

FIGURES

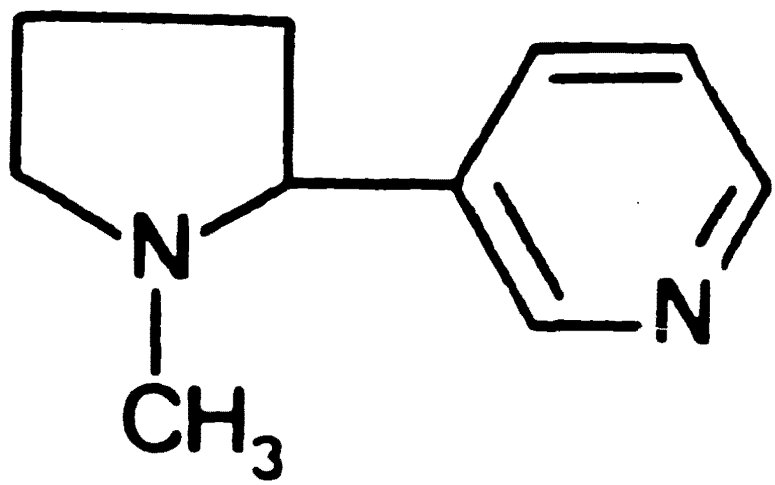


Figure 1. Chemical structure of nicotine.

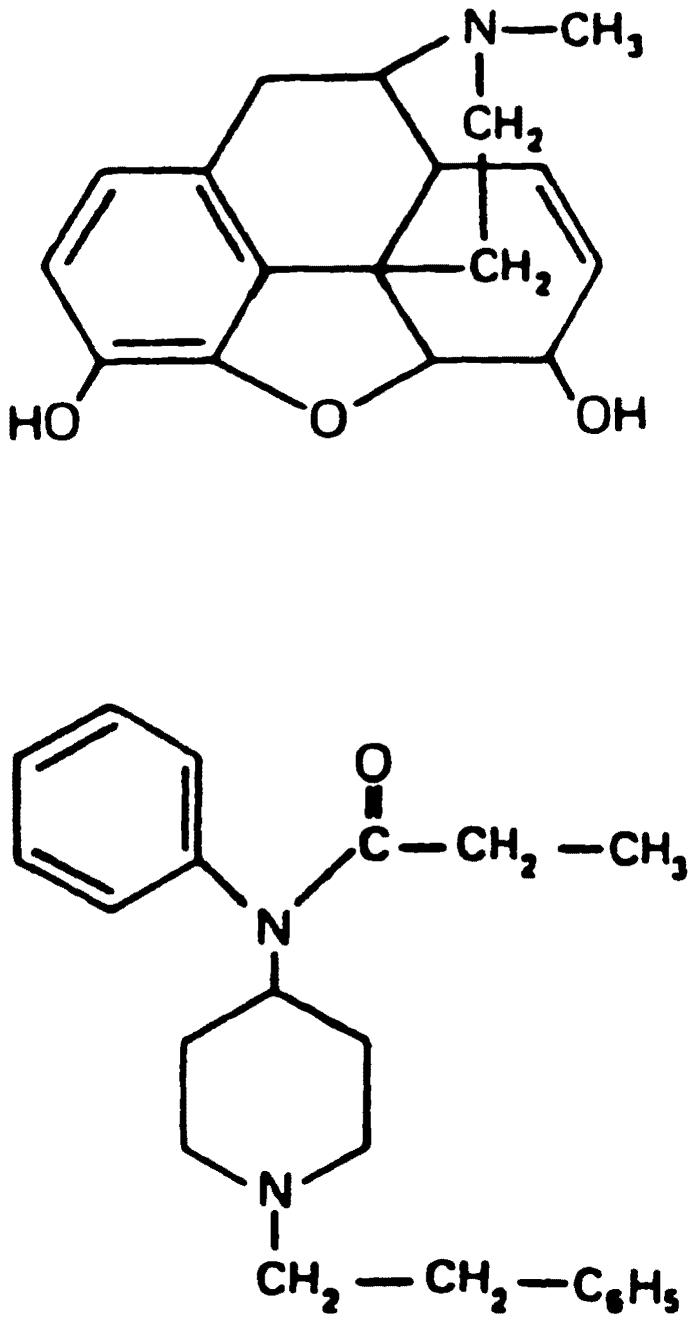


Figure 2. Chemical structure of morphine and fentanyl (two opioids that work at the μ receptor).

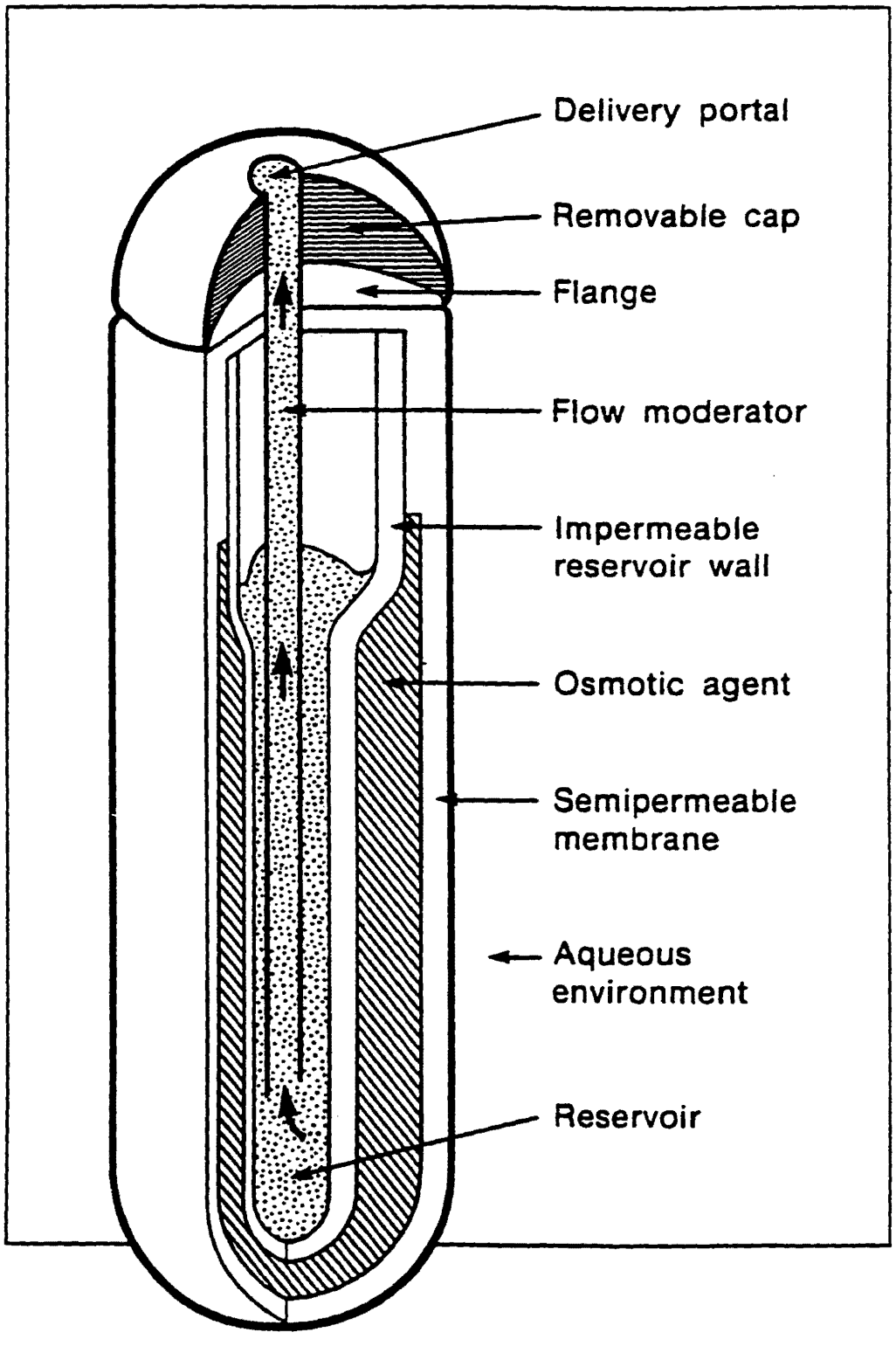


Figure 3. Diagram of Alzet® osmotic minipump (Model 2002; Alza Corporation).

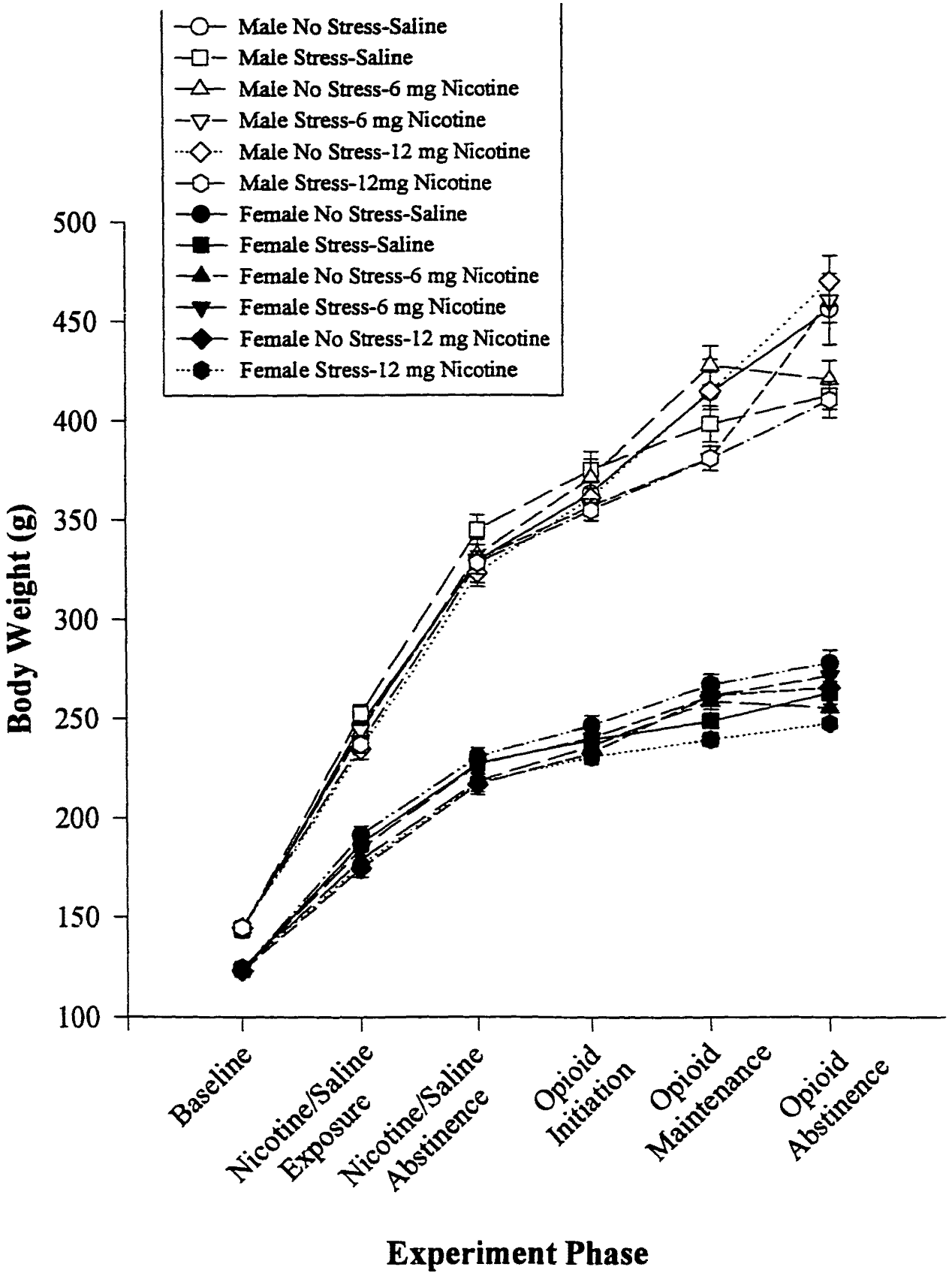


Figure 4. Average body weights (g) for all 12 treatment groups for each major phase of the experiment.

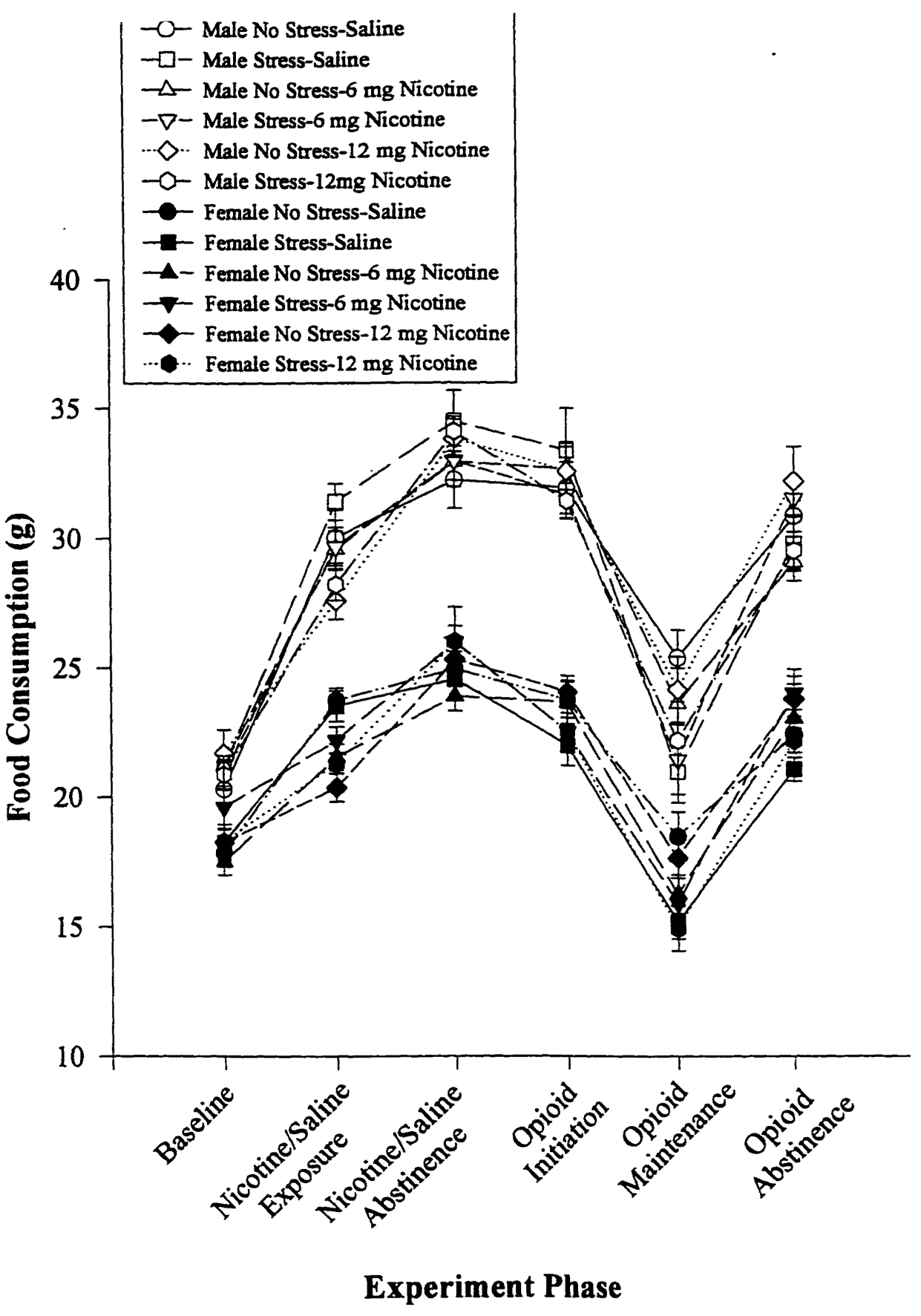


Figure 5. Average food consumption (g) for all 12 treatment groups for each major phase of the experiment.

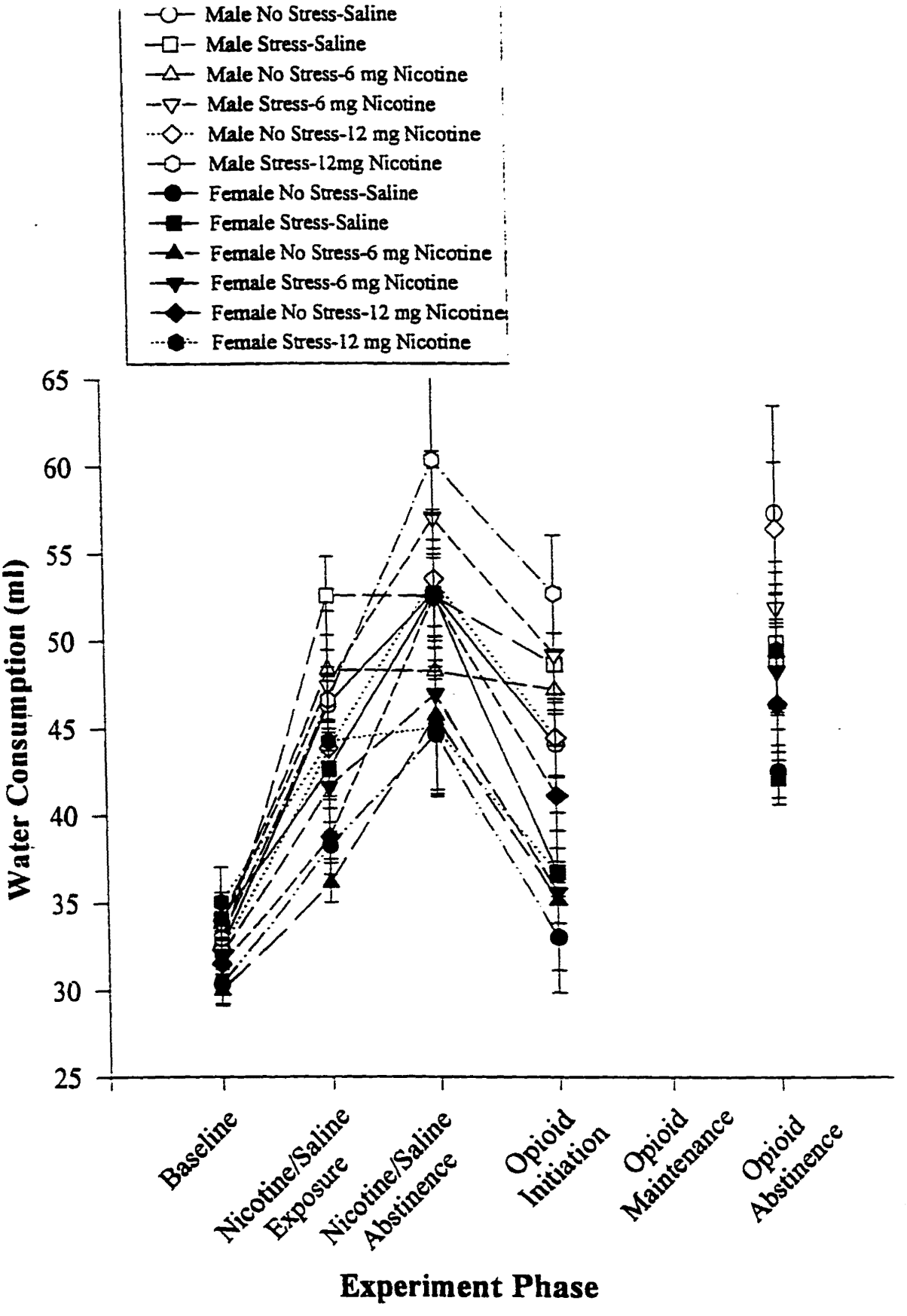


Figure 6. Average water consumption (ml) for all 12 treatment groups for each major phase of the experiment.

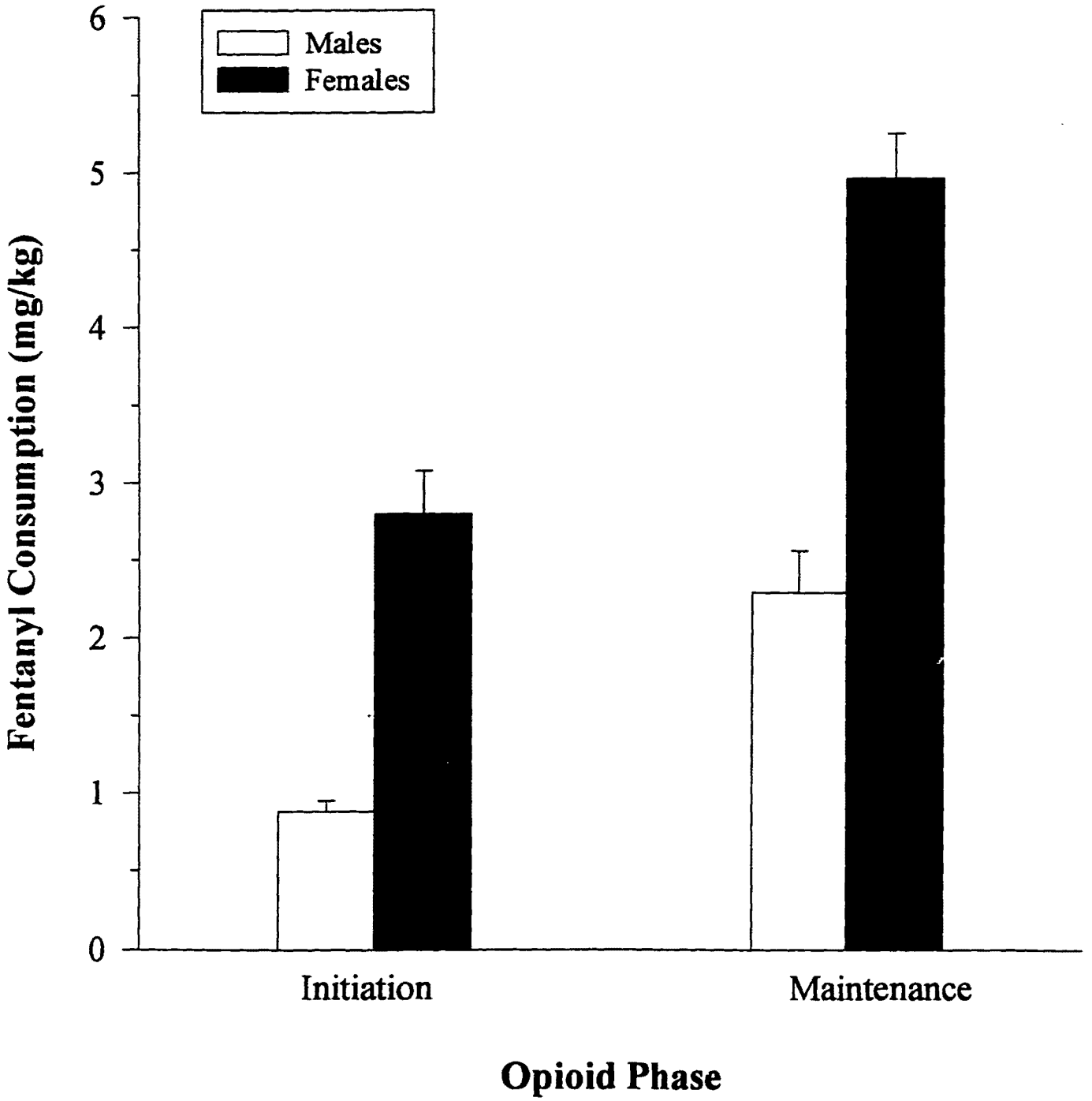


Figure 7. Fentanyl consumption (mg/kg) by male and female rats during initiation and maintenance.

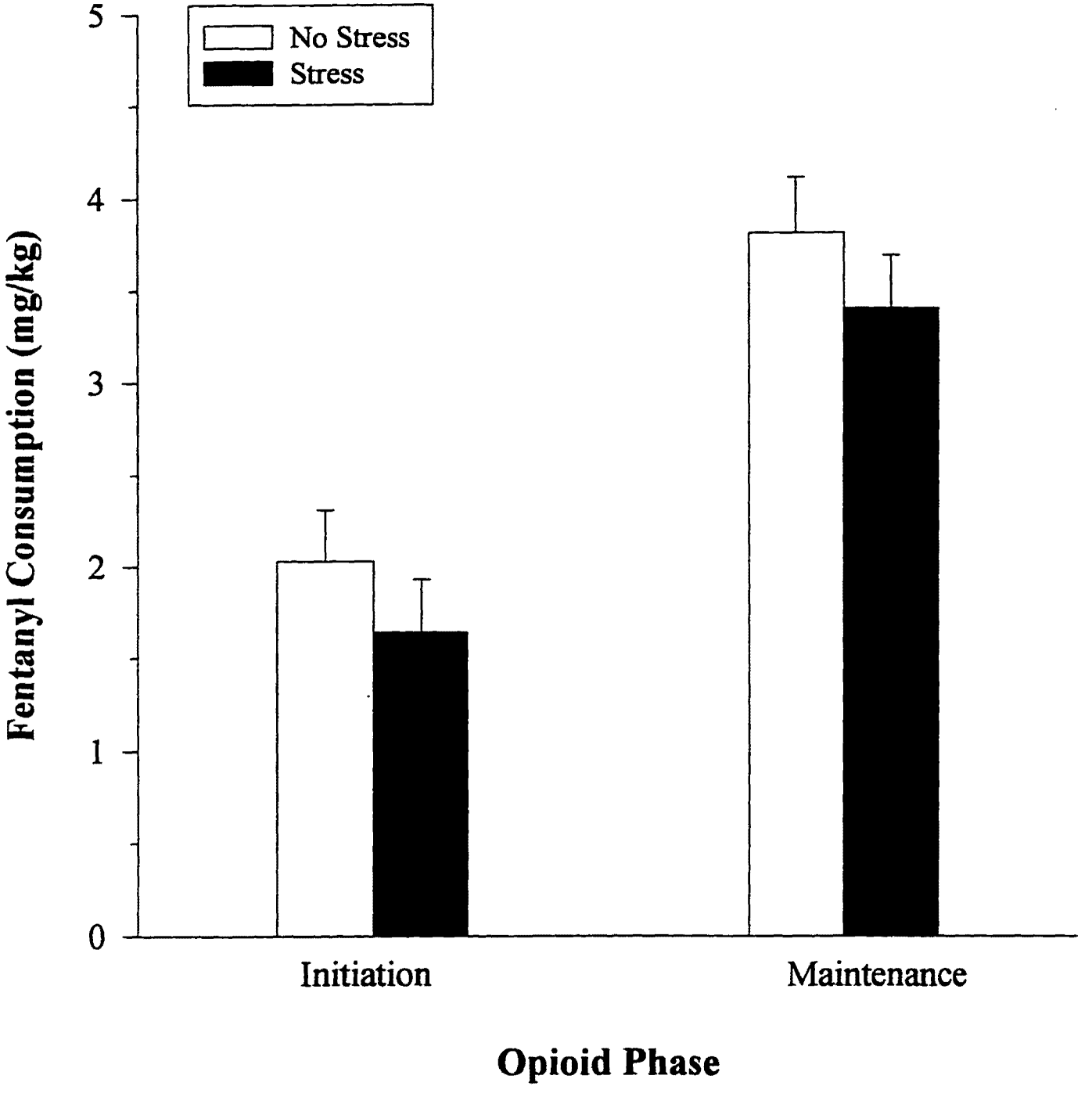


Figure 8. Fentanyl consumption (mg/kg) by no-stress and stress rats during initiation and maintenance.

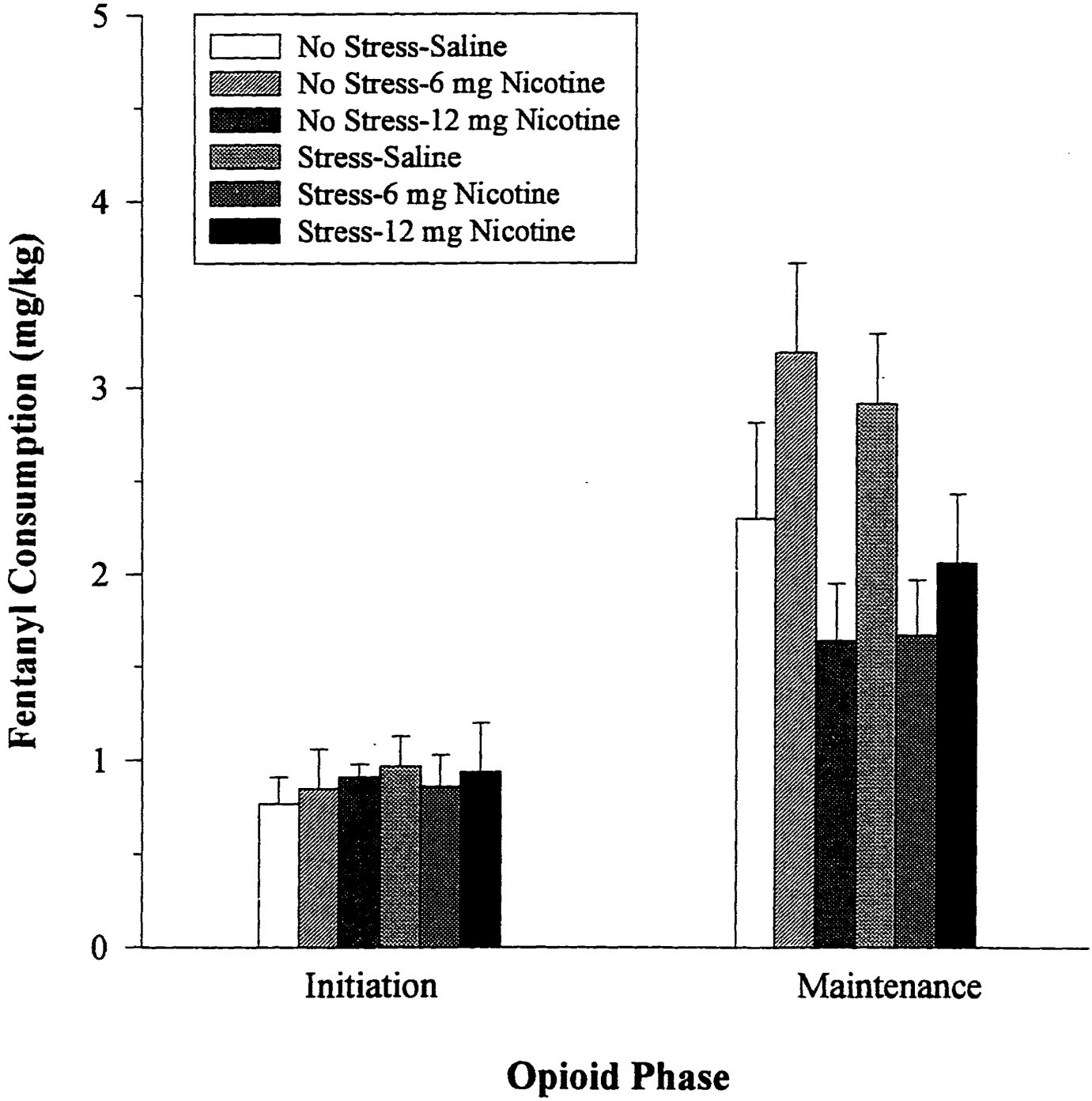


Figure 9. Fentanyl consumption (mg/kg) by no-stress and stress male rats exposed to saline, 6 mg nicotine/kg/day, or 12 mg nicotine/kg/day during initiation and maintenance.

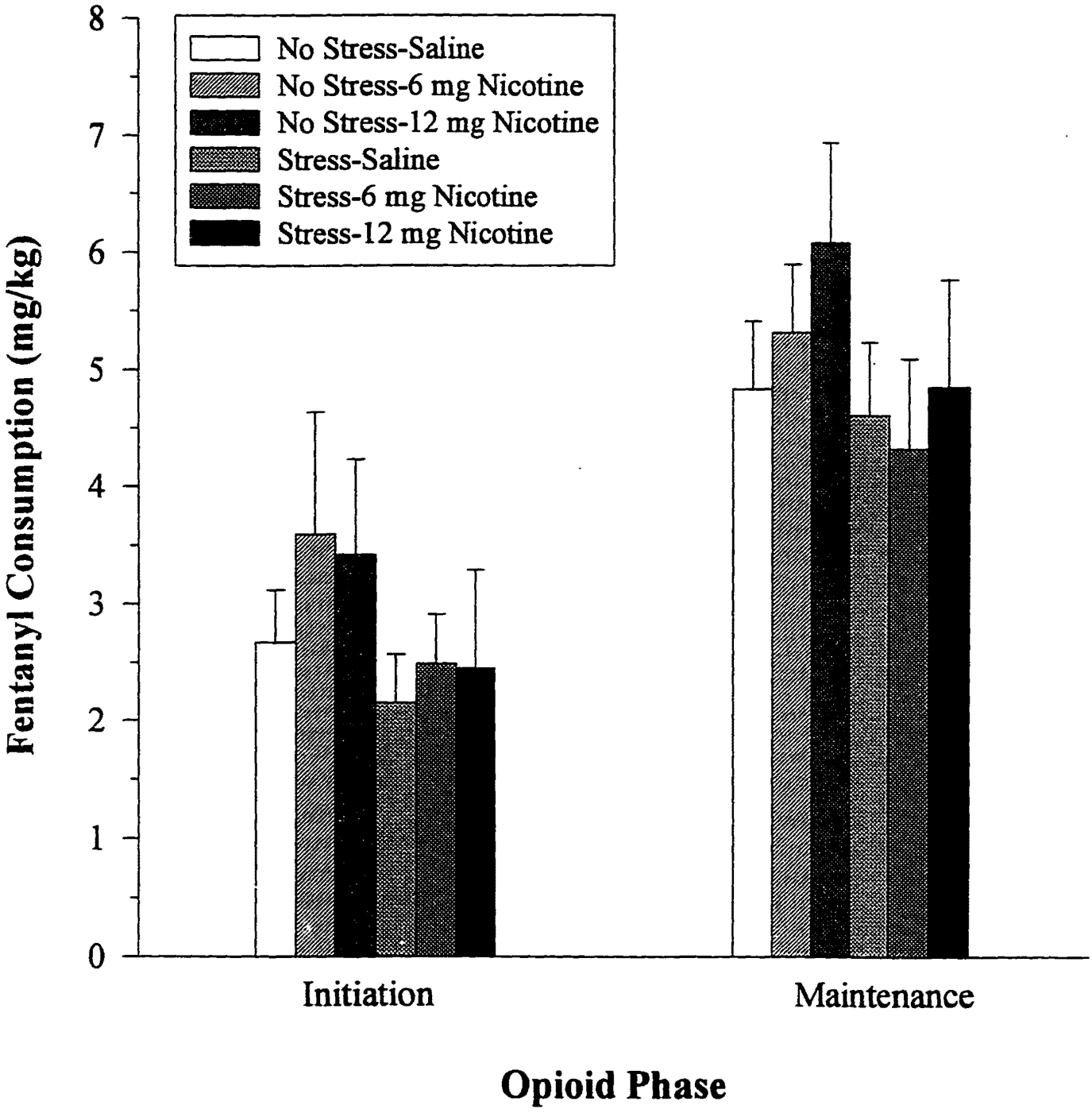


Figure 10. Fentanyl consumption (mg/kg) by no-stress and stress female rats exposed to saline, 6 mg nicotine/kg/day, or 12 mg nicotine/kg/day during initiation and maintenance.

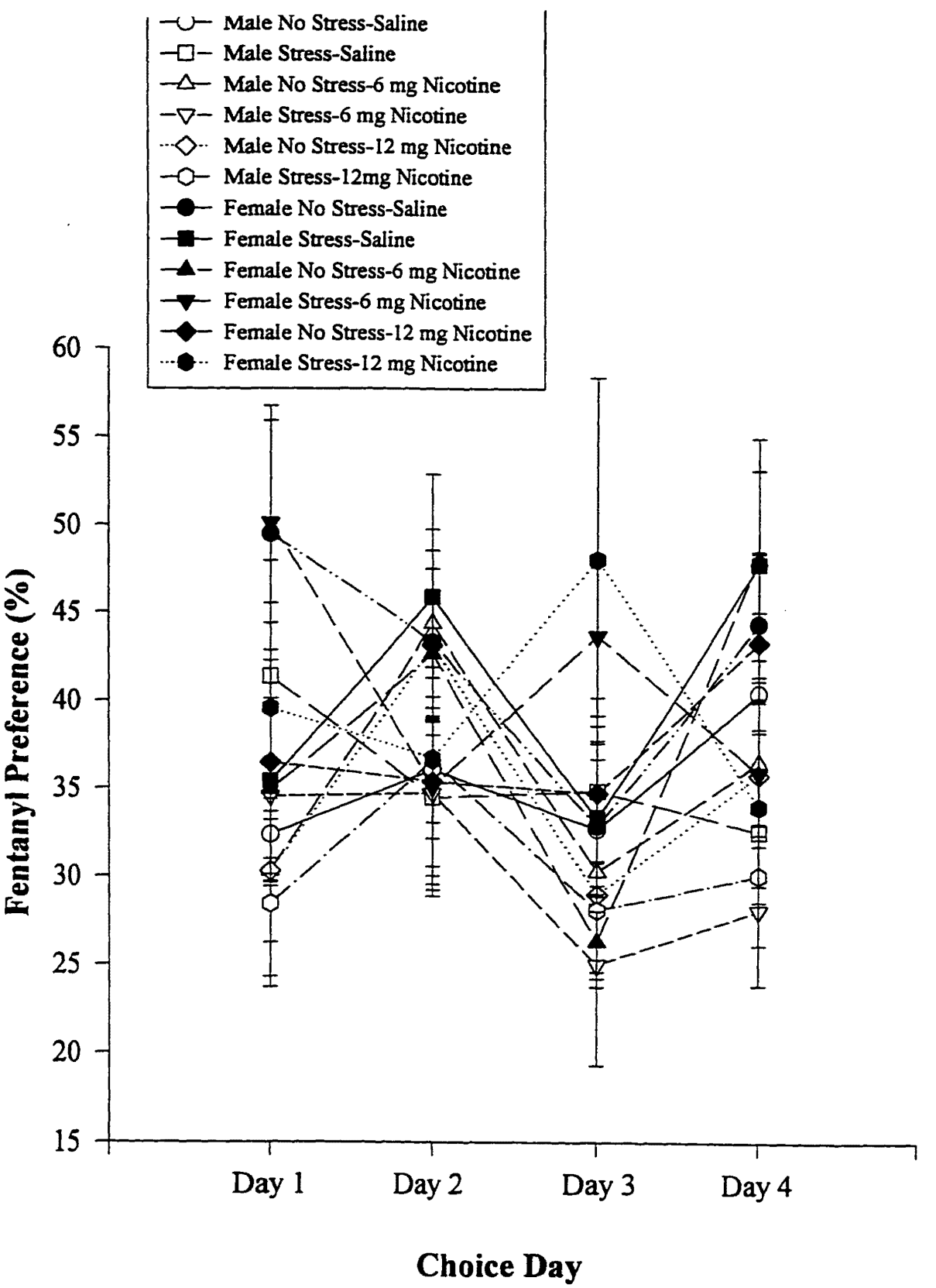


Figure 11. Fentanyl preference (percent) by no-stress and stress, male and female rats exposed to saline, 6 mg nicotine/kg/day, or 12 mg nicotine/kg/day for initiation choice days (4 days).

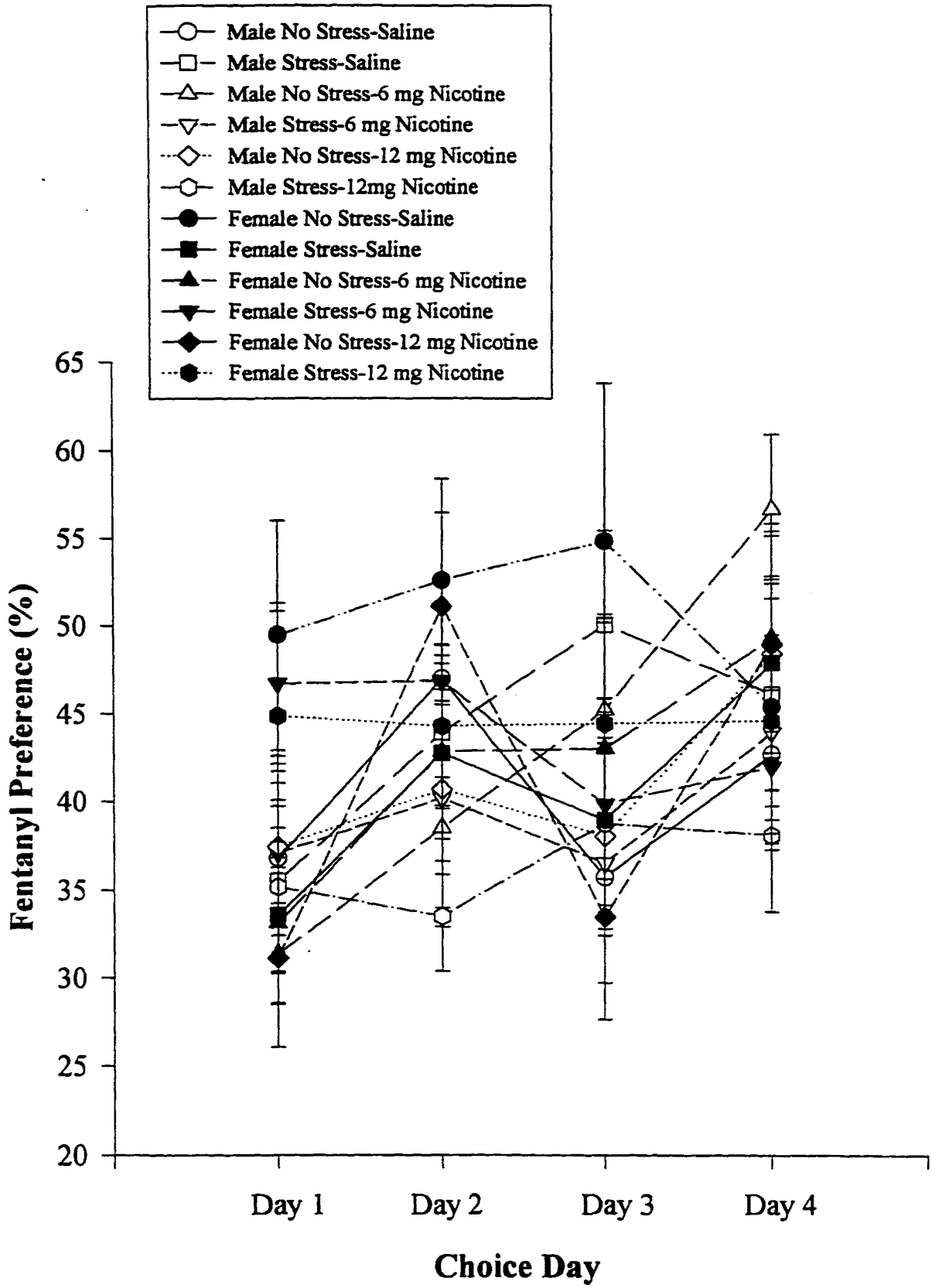


Figure 12. Fentanyl preference (percent) by no-stress and stress, male and female rats exposed to saline, 6 mg nicotine/kg/day, or 12 mg nicotine/kg/day for maintenance choice days (5 days).

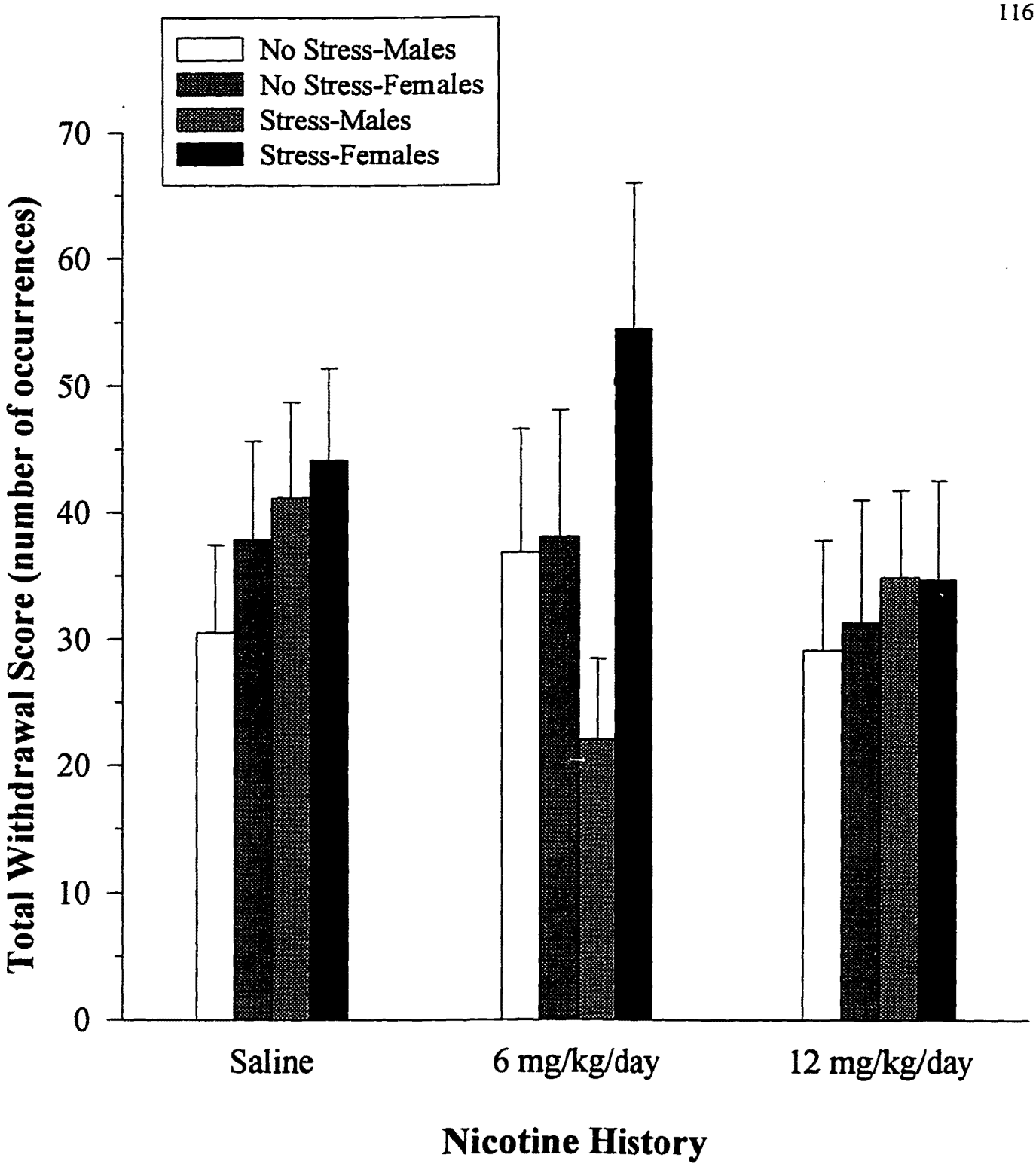


Figure 13. Mean withdrawal behaviors observed following naloxone injection in no-stress and stress male and female rats that were previously exposed to saline, 6 mg nicotine/kg/day, or 12 mg nicotine/kg/day.

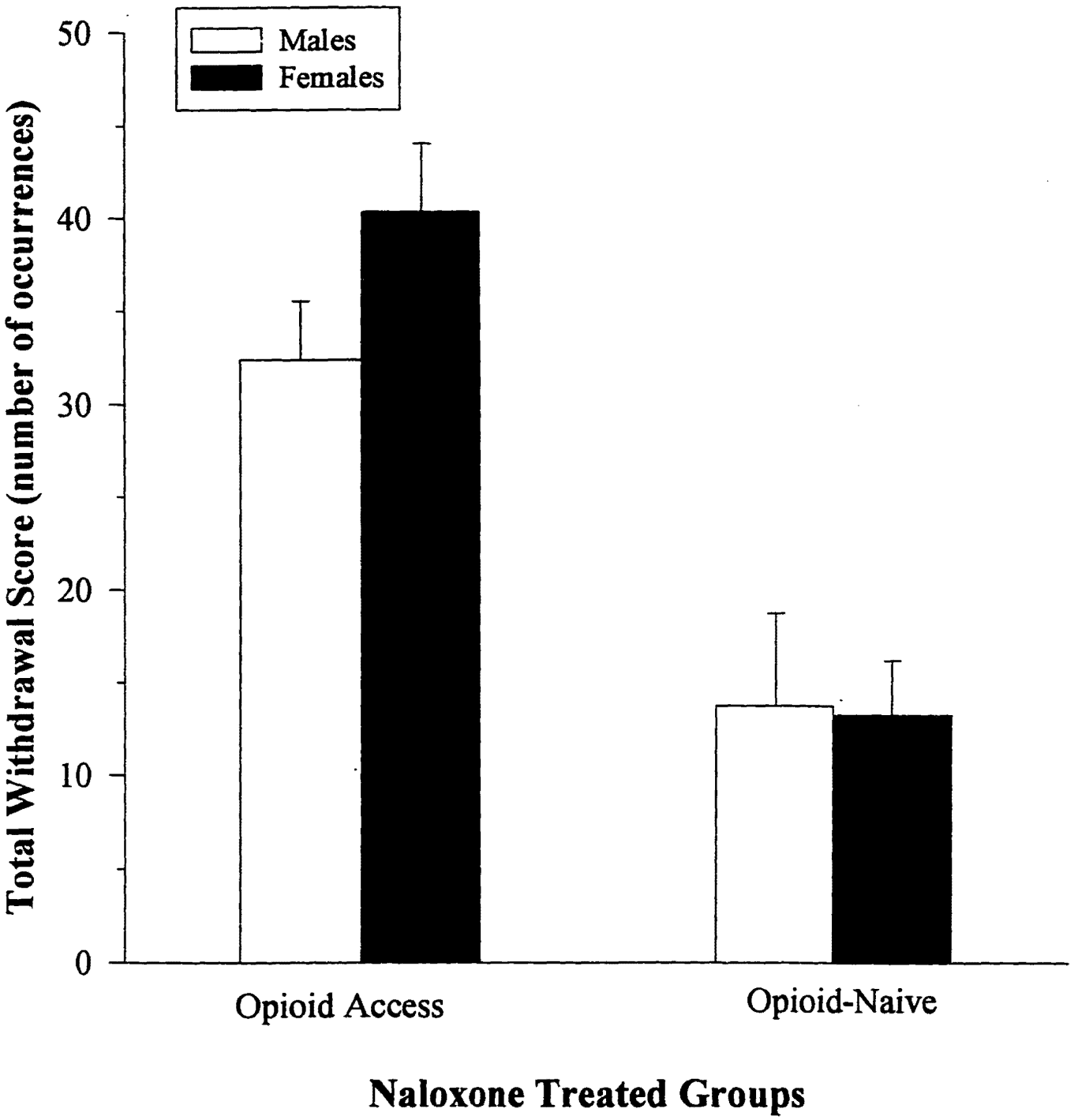


Figure 14. Mean withdrawal behaviors observed following naloxone injection in no-stress and stress male and female rats that were previously exposed to saline, 6 mg nicotine/kg/day, or 12 mg nicotine/kg/day and opioid/stress-naive control rats.

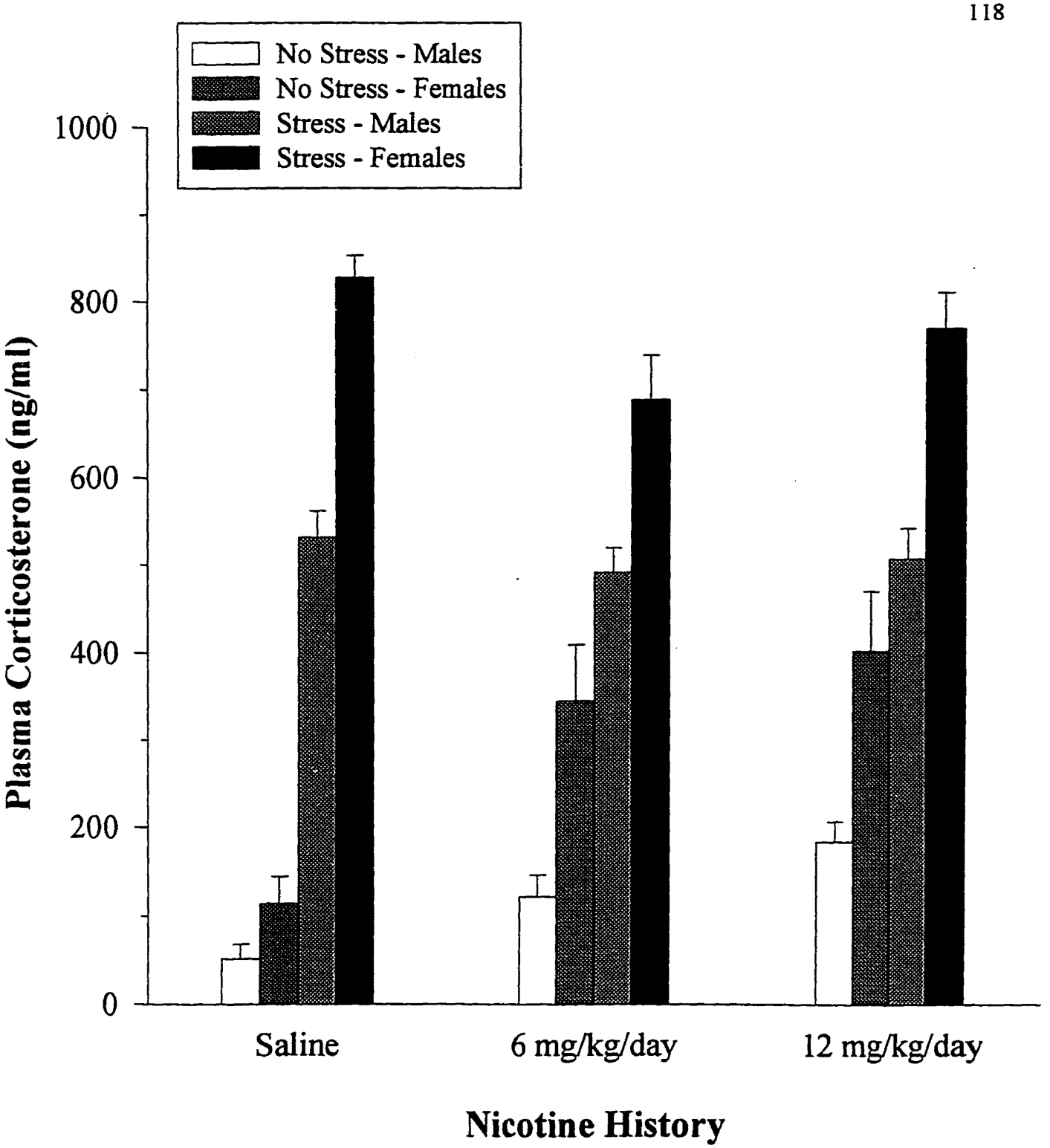


Figure 15. Plasma corticosterone levels (ng/ml) in saline or nicotine (6 or 12 mg nicotine/kg/day) exposed male and female rats on the last day of the experiment following either 20 minutes of immobilization stress or no-stress.

APPENDIX I

NICOTINE MINIPUMP CALCULATION SHEETS

- 6 mg Nicotine dihydrochloride/kg/day - Females
- 6 mg Nicotine dihydrochloride/kg/day - Males
- 12 mg Nicotine dihydrochloride/kg/day - Females
- 12 mg Nicotine dihydrochloride/kg/day - Males

NICOTINE CALCULATIONS FOR ALZET MINI-OSMOTIC PUMPS
(6 mg Nicotine dihydrochloride/kg/day - Females)

LOT #: **041106**

MEAN IN VITRO PUMP RATE= **0.48 µl of solution/hr**

MEAN FILL VOLUME= **237 µl of solution**

IN VIVO PUMP RATE CONVERSION = 0.9 (a constant for all Alzet minipumps)

(0.9) x (in vitro pump rate: **0.48µl/hr**) x (24 hr/day) = **10.368 µl solution/day**

NUMBER OF DAYS PUMP IS OPERABLE:

(pump fill volume: **237 µl**)/(**10.368 µl/day**) = **22.859 days**

DRUG DOSAGES:

(6 mg nicotine base/kg/day) x (1000 µl/ml) = **578.704 mg nicotine base/ml/kg**
(10.368 µl/day)

(*mean animal weight* in kg) x (**578.704 mg/ml/kg**) = _____mg nicotine free base/ml

(*nicotine free base* mg/ml) x (1.4494[1]) = _____mg nicotine dihydrochloride/ml

TOTAL DRUG SOLUTION VOLUME:

(pump fill volume: **237 µl**) x (number of animals: **20**) = **4740 ml** minimum volume drug solution

Prepare more than the minimum volume of drug solution

---

[1]CONVERSION FACTOR FOR NICOTINE FREE BASE:

$C_{10}H_{14}N_2$+2HCl MW = 235.13
$C_{10}H_{14}N_2$ MW = 162.23
(235.1)/(162.23) = 1.4494

NICOTINE CALCULATIONS FOR ALZET MINI-OSMOTIC PUMPS
(6 mg Nicotine dihydrochloride/kg/day - Males)

LOT #: **041106**

MEAN IN VITRO PUMP RATE= **0.48 µl of solution/hr**

MEAN FILL VOLUME= **237 µl of solution**

IN VIVO PUMP RATE CONVERSION = 0.9 (a constant for all Alzet minipumps)

(0.9) x (in vitro pump rate: **0.48µl/hr**) x (24 hr/day) = **10.368 µl solution/day**

NUMBER OF DAYS PUMP IS OPERABLE:

(pump fill volume: **237 µl**)/(**10.368 µl/day**) = **22.859 days**

DRUG DOSAGES:

(6 mg nicotine base/kg/day) x (1000 µl/ml) = **578.704 mg nicotine base/ml/kg**
(10.368 µl/day)

(*mean animal weight* in kg) x (**578.704 mg/ml/kg**) = _____mg nicotine free base/ml

(*nicotine free base* mg/ml) x (1.4494[1]) = _____mg nicotine dihydrochloride/ml

TOTAL DRUG SOLUTION VOLUME:

(pump fill volume: **237 µl**) x (number of animals: **20**) = **4740 ml** minimum volume drug solution

Prepare more than the minimum volume of drug solution

---

[1]CONVERSION FACTOR FOR NICOTINE FREE BASE:

$C_{10}H_{14}N_2$+2HCl MW = 235.13
$C_{10}H_{14}N_2$ MW = 162.23
(235.1)/(162.23) = 1.4494

NICOTINE CALCULATIONS FOR ALZET MINI-OSMOTIC PUMPS
(12 mg Nicotine dihydrochloride/kg/day - Females)

LOT #: **041106**

MEAN IN VITRO PUMP RATE= **0.48 µl of solution/hr**

MEAN FILL VOLUME= **237 µl of solution**

IN VIVO PUMP RATE CONVERSION = 0.9 (a constant for all Alzet minipumps)

(0.9) x (in vitro pump rate: **0.48µl/hr**) x (24 hr/day) = **10.368 µl solution/day**

NUMBER OF DAYS PUMP IS OPERABLE:

(pump fill volume: **237 µl**)/(**10.368 µl/day**) = **22.859 days**

DRUG DOSAGES:

(12 mg nicotine base/kg/day) x (1000 µl/ml) = **1157.407 mg nicotine base/ml/kg**
(10.368 µl/day)

(*mean animal weight* in kg) x (**1157.407 mg/ml/kg**) = _____mg nicotine free base/ml

(*nicotine free base* mg/ml) x (1.4494[1]) = _____mg nicotine dihydrochloride/ml

TOTAL DRUG SOLUTION VOLUME:

(pump fill volume: **237 µl**) x (number of animals: **20**) = **4740 ml** minimum volume drug solution

Prepare more than the minimum volume of drug solution

---

[1]CONVERSION FACTOR FOR NICOTINE FREE BASE:

$C_{10}H_{14}N_2$+2HCl MW = 235.13
$C_{10}H_{14}N_2$ MW = 162.23
(235.1)/(162.23) = 1.4494

NICOTINE CALCULATIONS FOR ALZET MINI-OSMOTIC PUMPS
(12 mg Nicotine dihydrochloride/kg/day - Males)

LOT #: **041106**

MEAN IN VITRO PUMP RATE= **0.48 µl of solution/hr**

MEAN FILL VOLUME= **237 µl of solution**

IN VIVO PUMP RATE CONVERSION = 0.9 (a constant for all Alzet minipumps)

(0.9) x (in vitro pump rate: **0.48µl/hr**) x (24 hr/day) = **10.368 µl solution/day**

NUMBER OF DAYS PUMP IS OPERABLE:

(pump fill volume: **237 µl**)/(**10.368 µl/day**) = **22.859 days**

DRUG DOSAGES:

(12 mg nicotine base/kg/day) x (1000 µl/ml) = **1157.407 mg nicotine base/ml/kg**
(10.368 µl/day)

(*mean animal weight* in kg) x (**1157.407 mg/ml/kg**) = _____mg nicotine free base/ml

(*nicotine free base* mg/ml) x (1.4494[1]) = _____mg nicotine dihydrochloride/ml

TOTAL DRUG SOLUTION VOLUME:

(pump fill volume: **237 µl**) x (number of animals: **20**) = **4740 ml** minimum volume drug solution

Prepare more than the minimum volume of drug solution

---

[1]CONVERSION FACTOR FOR NICOTINE FREE BASE:

$C_{10}H_{14}N_2$+2HCl MW = 235.13
$C_{10}H_{14}N_2$ MW = 162.23
(235.1)/(162.23) = 1.4494

APPENDIX II

STRESSOR TREATMENT AND OPIOID AVAILABILITY SCHEDULE

Stress and No-Stress Group Assignments

Group	Female No-Stress	Female Stress	Male No-Stress	Male Stress
Group A	Saline (n=3) 6 mg nic (n=3) 12 mg nic (n=4)	Saline (n=4) 6 mg nic (n=3) 12 mg nic (n=3)		
Group B	Saline (n=4) 6 mg nic (n=3) 12 mg nic (n=3)	Saline (n=3) 6 mg nic (n=4) 12 mg nic (n=3)		
Group C	Saline (n=3) 6 mg nic (n=4) 12 mg nic (n=3)	Saline (n=3) 6 mg nic (n=3) 12 mg nic (n=4)		
Group D			Saline (n=3) 6 mg nic (n=3) 12 mg nic (n=4)	Saline (n=4) 6 mg nic (n=3) 12 mg nic (n=3)
Group E			Saline (n=4) 6 mg nic (n=3) 12 mg nic (n=3)	Saline (n=3) 6 mg nic (n=4) 12 mg nic (n=3)
Group F			Saline (n=3) 6 mg nic (n=4) 12 mg nic (n=3)	Saline (n=3) 6 mg nic (n=3) 12 mg nic (n=4)

Stressor Ordering & Opioid Availability Schedule

Order	1st Group	2nd Group	3rd Group	4th Group	5th Group	6th Group
Order 1	**A**	**B**	**C**	**D**	**E**	**F**
Order 2	**B**	**C**	**D**	**E**	**F**	**A**
Order 3	**C**	**D**	**E**	**F**	**A**	**B**
Order 4	**D**	**E**	**F**	**A**	**B**	**C**
Order 5	**E**	**F**	**A**	**B**	**C**	**D**
Order 6	**F**	**A**	**B**	**C**	**D**	**E**

APPENDIX III

NALOXONE CHALLENGE TREATMENT SCHEDULE

- DAY 1
- DAY 2

NALOXONE CHALLENGE SCHEDULE: DAYS 1 & 2

G130 = Animal housing room
G150 = Stressor treatment room
G151 = Naloxone behavioral observation room
G125 = Weighing room
G114 = Naloxone injection room

7:00 Group F-Str to G150

7:05 Begin stressor

7:10 Group F-NS receives bottles

7:25 End stressor

7:35 Group F-St receives bottles

7:50 Group A-St to G150

7:55 Begin stressor

8:00 Group A-NS receives bottles

8:15 End stressor

8:25 Group A-St receives bottles

8:40 Group D-St to G150

8:45 Begin stressor

8:50 Group D-NS receives bottles

9:05 End stressor

9:15 Group D-St receives bottles

9:30 Group B-St to G150

9:35 Begin stressor

9:40 Group B-NS receives bottles

9:55 End stressor

10:05 Group B-St receives bottles

10:20 Group E-St to G150

10:25 Begin stressor

10:30 Group E-NS receives bottles

10:45 End stressor

10:55 Group E-St receives bottles

11:10 Group C-St to G150

11:15 Begin stressor

11:20 Group C-NS receives bottles

11:35 End stressor

11:45 Group C-St receives bottles

1:10 Pull Group F-NS bottles and deliver to G125

Weigh Group F-NS and deliver naloxone rats to G114

1:20 Inject Group F-NS naloxone rats and deliver to G151

1:30 Begin behavioral observations on Group F-NS

1:35 Pull Group F-St bottles and deliver to G125

Weigh Group F-St and deliver naloxone rats to G114

1:45 Inject Group F-St naloxone rats and deliver to G151

1:50 Pick up Group F-NS & deliver to G125 for Post-BWT; return rack to G130

1:55 Begin behavioral observations on Group F-St

2:00 Pull Group A-NS bottles and deliver to G125

Weigh Group A-NS and deliver naloxone rats to G114

2:10 Inject Group A-NS naloxone rats and deliver to G151

2:15 Pick up Group F-St & deliver to G125 for Post-BWT; return rack to G130

2:20 Begin behavioral observations on Group A-NS;

2:25 Pull Group A-St bottles and deliver to G125

Weigh Group A-St and deliver naloxone rats to G114

2:35 Inject Group A-St naloxone rats and deliver to G151

2:40 Pick up Group A-NS & deliver to G125 for Post-BWT; return rack to G130

2:45 Begin behavioral observations on Group A-St

2:50 Pull Group D-NS bottles and deliver to G125

Weigh Group D-NS and deliver naloxone rats to G114

3:00 Inject Group D-NS naloxone rats and deliver to G151

3:05 Pick up Group A-St & deliver to G125 for Post-BWT; return rack to G130

3:10 Begin behavioral observations on Group D-NS;

3:15 Pull Group D-St bottles and deliver to G125

Weigh Group D-St and deliver naloxone rats to G114

3:25 Inject Group D-St naloxone rats and deliver to G151

3:30 Pick up Group D-NS & deliver to G125 for Post-BWT; return rack to G130

3:35 Begin behavioral observations on Group D-St

3:40 Pull Group B-NS bottles and deliver to G125

Weigh Group B-NS and deliver naloxone rats to G114

3:50 Inject Group B-NS naloxone rats and deliver to G151

3:55 Pick up Group D-St & deliver to G125 for Post-BWT; return rack to G130

4:00 Begin behavioral observations on Group B-NS;

4:05 Pull Group B-St bottles and deliver to G125

Weigh Group B-St and deliver naloxone rats to G114

4:15 Inject Group B-St naloxone rats and deliver to G151

4:20 Pick up Group B-NS & deliver to G125 for Post-BWT; return rack to G130

4:25 Begin behavioral observations on Group B-St

4:30 Pull Group E-NS bottles and deliver to G125

Weigh Group E-NS and deliver naloxone rats to G114

4:40 Inject Group E-NS naloxone rats and deliver to G151

4:45 Pick up Group B-St & deliver to G125 for Post-BWT; return rack to G130

4:50 Begin behavioral observations on Group E-NS;

4:55 Pull Group E-St bottles and deliver to G125

Weigh Group E-St and deliver naloxone rats to G114

5:05 Inject Group E-St naloxone rats and deliver to G151

5:10 Pick up Group E-NS & deliver to G125 for Post-BWT; return rack to G130

5:15 Begin behavioral observations on Group E-St

5:20 Pull Group C-NS bottles and deliver to G125

Weigh Group C-NS and deliver naloxone rats to G114

5:30 Inject Group C-NS naloxone rats and deliver to G151

5:35 Pick up Group E-St & deliver to G125 for Post-BWT; return rack to G130

5:40 Begin behavioral observations on Group C-NS;

5:45 Pull Group C-St bottles and deliver to G125

Weigh Group C-St and deliver naloxone rats to G114

5:55 Inject Group C-St naloxone rats and deliver to G151

6:00 Pick up Group C-NS & deliver to G125 for Post-BWT; return rack to G130

6:05 Begin behavioral observations on Group C-St

6:25 Pick up Group C-St and return to housing room (G130)

APPENDIX IV

NALOXONE WITHDRAWAL OBSERVATION DATA SHEET

WITHDRAWAL SYMPTOMS OBSERVATION DATA SHEET

Body Weight: Pre_____ Post_____ Change_____

Naloxone Dosage:_____ml _____mg/kg

1. **Wet-dog shakes** (# of times):

_____

2. **Diarrhea** (# of times):

_____

3. **Mouthing and teeth chattering** (# of times):

_____

4. **Ptosis** (# of times):

_____

5. **Excessive grooming** (# of times):

_____

6. **Abnormal posture** (# of times):

_____

Observer Initials:_____ Date:_____ Time:_____

Subject#:_____ **TOTAL SCORE:_____**

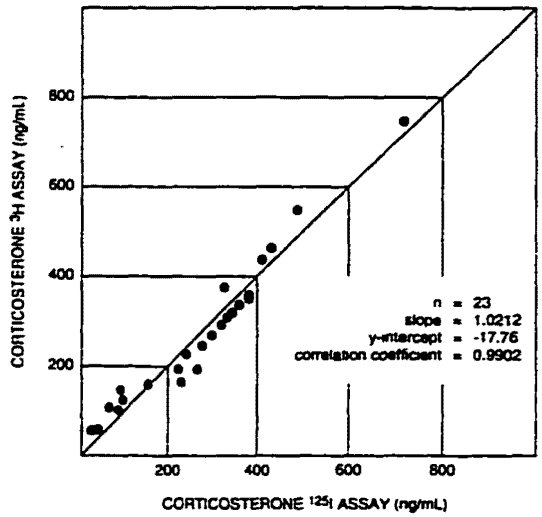
APPENDIX V

PACKAGE INSERT FOR IMMUCHEM™ DOUBLE ANTIBODY

CORTICOSTERONE RIA KIT FOR RATS AND MICE

D. ASSAY CORRELATION

Twenty three laboratory rat serum samples covering the entire physiological range were assayed with ImmuChem™ CpB-^{3}H kit method requiring heat denaturation and ImmuChem™ CpB-^{125}I kit method described herein. The following data were obtained:



XVI. SPECIFICITY OF THE ANTISERUM

The following materials have been checked for cross reactivity. The percentages indicate cross reactivity at 50% displacement compared to corticosterone.

Steroids	% Cross Reaction
Corticosterone	100.00
Desoxycorticosterone	0.34
Testosterone	0.10
Cortisol	0.05
Aldosterone	0.03
Progesterone	0.02
Androstenedione	0.01
5α-Dihydrotestosterone	0.01
Cholesterol	<0.01
Dehydroepiandrosterone	<0.01
Dehydroepandrosterone-sulfate	<0.01
11-Desoxycortisol	<0.01
20α-Dihydroprogesterone	<0.01
Estrone	<0.01
Estradiol-17α	<0.01
Estradiol-17β	<0.01
Estriol	<0.01
Pregnenolone	<0.01
17α-Hydroxypregnenolone	<0.01
17α-Hydroxyprogesterone	<0.01

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[redacted]

ImmuChem™ Double Antibody

Corticosterone

^{125}I RIA Kit

For Rats and Mice

For Research Use Only

ICN Biomedicals, Inc.
Diagnostics Division
3300 Hyland Avenue
Costa Mesa, CA 92626

ICN

814201
REV. D (7-94)

IMMUCHEM™ DOUBLE ANTIBODY
CORTICOSTERONE
RIA KIT
FOR RATS AND MICE

** NOTICE: LYOPHILIZED RAT CONTROL SET**

In order to maximize the stability of the ImmuChem™ rat control set for corticosterone, this control set will be sent out in predituted, lyophilized form.

To prepare the control set for ^{125}I corticosterone assay, reconstitute each level with 2.0 mL of distilled water, and allow to sit at room temperature for at least 30 minutes.

After reconstitution, assay directly; control has been predituted 1:200. DO NOT FURTHER DILUTE.

FOR INVESTIGATIONAL USE ONLY

I. INTRODUCTION

A. Intended Use

The ImmuChem™ ^{125}I Corticosterone RIA is specifically designed for use in laboratory mice and rats. Utilizing the combination of a highly specific antiserum for corticosterone and a proprietary blocking function incorporated in the assay system, this RIA is suitable for determination of corticosterone in unextracted serum or plasma without a protein denaturation step. Also the use of an ^{125}I label eliminates the need for the liquid scintillation counting step required with tritiated assays.

B. Physiology

Corticosterone is the principle glucocorticoid secreted by the adrenal cortices of mice and rats.[1] Secretion of corticosterone in these species is modulated by a complex negative feedback mechanism involving the central nervous system, hypothalamus, pituitary, and adrenals. ACTH released from the pituitary augments adrenal secretion of corticosterone while falling levels of corticosterone are associated with rising levels of ACTH.[3] In both mice and rats there is a circadian rhythm of corticosterone release with the highest concentrations being observed between 1600 and 2200 hours in a normal laboratory environment.[2]

C. Laboratory Applications

Corticosterone measurements are a useful index of general and neuroendocrine response to the stress of laboratory experiments in mice and rats. Thus corticosterone concentrations rise sharply in healthy, intact animals following exposure to experimental stimuli such as drugs,[1] barometric shock,[3] experimental disease state,[4] or abrupt temperature shifts.[1] and may serve to document the neuroendocrine and endocrine integrity of the preparation while observations are being made.[3-5]

II. PRINCIPLE OF TEST

Radioimmunoassay (RIA) is the term applied to the measurement of the concentration of antigen molecules using a radioactive label that quantitates the amount of antigen (i.e., hormone) by determination of the extent to which it combines with its antibody.

In the assay, a limited amount of specific antibody (Ab) is reacted with the corresponding hormone ($^*H$) labeled with a radioisotope. Upon addition of an increasing amount of the hormone (H), a correspondingly decreasing fraction of $^*H$ added is bound to the antibody. After separation of the bound from the free $^*H$ by various means, the amount of radioactivity in one or both of these two fractions is evaluated and used to construct a standard curve against which the unknown samples are measured.

III. REAGENTS PROVIDED AND LABEL COLOR CODE (100 Tube Kit)

COMPONENT	LABEL COLOR	VOLUME
Steroid Diluent Cat # 07-156196	white	150 mL
Anti-Corticosterone Cat # 07-120113	yellow	22 mL
Calibrators (25-1000 ng/mL) Cat # 07-120130	green	2.0 mL ea
Precipitant Solution Cat # 07-156624	red	56 mL
Corticosterone-^{125}I Cat # 07-120121	blue	22 mL
Corticosterone Controls Cat # 07-120180	beige	0.1 mL ea

IV. REAGENTS DESCRIPTION AND PREPARATION (FOR IN-VITRO DIAGNOSTIC USE)

NOTES:

(1) The concentrations of calibrators are expressed in terms of serum equivalence. To obtain actual concentration of corticosterone in ng/mL, divide calibrator value by 200 (serum dilution ratio, 1:200).

(2) To prepare the control set for corticosterone assay, reconstitute each level with 2.0 mL of distilled water, and allow to sit at room temperature for at least 30 minutes.

(3) After reconstitution, assay directly; control has been predituted 1:200. DO NOT FURTHER DILUTE.

All reagents (except controls) are shipped ready to use.

A. STEROID DILUENT

Phosphosaline gelatin buffer (pH 7.0±0.1) containing rabbit gamma globulins.

STABILITY: *Refer to expiration date on kit vial.*
STORAGE: *2-8°C.*

B. ANTI-CORTICOSTERONE

Corticosterone-3-carboxymethyloxime: BSA was used as the antigen to generate antiserum in rabbits. The antiserum is titered to bind 50-60% of the corticosterone-^{125}I derivative in the absence of nonradioactive corticosterone.

STABILITY: *Refer to expiration date on kit vial.*
STORAGE: *2-8°C.*

C. CORTICOSTERONE CALIBRATORS

See Note (1): Six calibrators are provided at the following concentrations: 25 ng/mL, 50 ng/mL, 100 ng/mL, 250 ng/mL, 500 ng/mL and 1000 ng/mL. The calibrators have been diluted with Steroid Diluent (A).

STABILITY: *Refer to expiration date on kit vial.*
STORAGE: *2-8°C.*

D. PRECIPITANT SOLUTION

This is a mixture of PEG and Goat anti-rabbit gamma globulins contained in TRIS buffer. 0.5 mL of this precipitant will immediately precipitate all the antibody bound antigen.

STABILITY: *Refer to expiration date on kit vial.*
STORAGE: *2-8°C.*

E. CORTICOSTERONE-^{125}I DERIVATIVE

This radioactive material contains less than 7 μCi per vial for a 200 tube kit and less than 3.5 μCi per vial for a 100 tube kit on the date of shipment. 0.2 mL of this radioactive derivative will provide approximately 50,000 cpm at 75% counter efficiency on the date of shipment.

STABILITY: *Refer to expiration date on vials.*
STORAGE: *2-8°C.*

V. LIMITATIONS, PRECAUTIONS AND GENERAL COMMENTS

A. Care must be taken that samples contain no exogenous radioactivity since its presence may lead to erroneous results.

B. The reagents provided in this kit are intended only for the specific quantitation of serum or plasma corticosterone in rats or mice. While specific quantitation of corticosterone in other animal species may be possible, its use in these systems should be independently determined by the user.

THIS KIT IS <u>NOT</u> TO BE USED TO DETERMINE CORTICOSTERONE IN HUMANS.

C. Strict adherence to the protocol is recommended. Any changes should be done at the discretion of the user.

D. The reagents provided in this kit are for the quantitation of corticosterone in rats and mice only.

E. The kit reagents and materials are intended for use as an integral unit. Do not mix various lots of any component reagent within an individual run.

F. RADIOACTIVE MATERIAL HANDLING

Please observe the following precautions when handling this radioactive material.

(1) This radioactive material may be received, acquired, possessed, and used only by physicians, clinical labs, or hospitals, and is intended for in-vitro laboratory tests not involving internal or external administration of the materials. Thus, the possession, use and transfer of the radiation herein are subject to the regulations of, and with a general license from, the U.S. NRC or the State with which the NRC has entered into agreement for the exercise of regulatory authority.

(2) Immediately upon receipt of this kit, check for breakage and verify contents as per the packing list. Should there be breakage or questions regarding this kit's contents, please contact your ICN representative or the ICN Technical Service Department (800) 854-0530.

(3) Kit reagents should be stored and used only at clean, designated work stations of the laboratory. Although exposure to radiation from the small amount of radioactive material supplied is negligible, it is good practice to designate a storage area at least 10 feet from any work station. Furthermore, persons under the age of 18 should not be permitted to handle radioactive material or enter an area where it is present.

(4) Should there be spillage of any radioactive material, the following clean-up procedure is recommended. While wearing rubber gloves, blot the spillage with a paper towel. This contaminated towel should be disposed as radioactive waste. Wash the affected area with a detergent, then rinse gloves with water, tear to prevent further use and discard as ordinary waste.

(5) The pipetting of radioactive material by mouth should not be permitted. Smoking, eating, or drinking while performing tests involving radioactive material should be prohibited. Lastly, persons handling radioactive material should wash their hands immediately after handling and prior to leaving the laboratory.

VI. SPECIMEN COLLECTION AND HANDLING

A significant increase in the baseline corticosterone levels is observed when animals are agitated prior to the collection of a blood sample. As a consequence, it is desirable that animals be routinely handled to alleviate this problem.

Plasma or serum can be used for the assay. They should be stored frozen (below -15°C), unless they are to be analyzed within a 48 hour period.

VII. EQUIPMENT AND REAGENTS REQUIRED BY THE USER

In addition to the reagents supplied with the kit, the following materials are required:

* (1) Pipettor and/or pipets that can accurately and precisely deliver the required volumes.
* (2) Gamma counter
(3) Laboratory vortex mixer.
(4) Test tube rack.
(5) Centrifuge-refrigerated (preferred) capable of 2300-2500 rpm (1000 x g).
(6) 10 x 75 mm tubes for RIA.
(7) Absorbent paper for blotting.

*Available from ICN Biomedicals, Inc. - (800) 854-0530

VIII. ASSAY PROCEDURE

A. ASSAY PREPARATIONS

(1) Bring reagents to room temperature prior to use.
(2) Set up assay in consecutively numbered 10 x 75 mm glass test tubes.
(3) Add solutions in the order indicated in the protocol. Pipet all reagents directly from shipping vials.

B. ASSAY STEPS

(1) Dilute rat or mouse serum 1:200 with STEROID DILUENT by taking 10 µL of sample to 2.0 mL.

(2) Add 0.3 mL STEROID DILUENT to tubes 1 and 2 (NSB tubes).

(3) Add 0.1 mL STEROID DILUENT to tubes 3 and 4 (0 tubes).

(4) Add 0.1 mL CORTICOSTERONE CALIBRATORS* (25 ng/mL- 1000 ng/mL) to tubes 5 thru 16.

NOTE

*Concentration of calibrators are expressed as serum equivalent and have already included the dilution factor. Results can be read directly from the calibration curve if our recommended dilution (1:200) is followed.

(5) Add 0.1 mL DILUTED (1:200) CONTROLS and DILUTED (1:200) RAT/MOUSE SERUM to tubes 17 to end of assay.

(6) Add 0.2 mL CORTICOSTERONE-^{125}I (blue reagent) to all tubes.

NOTE

^{125}I TRACER MUST BE ADDED BEFORE ANTI-SERUM.

(7) Add 0.2 mL ANTI-CORTICOSTERONE (yellow reagent) to tubes 3 to end of assay.

NOTE

DO NOT ADD ANTISERUM TO TUBES 1 AND 2.

(8) Vortex mix all assay tubes and incubate at room temperature (22°-25°C) for 2 hours.

(9) After incubation, add 0.5 mL PRECIPITANT SOLUTION (red reagent) to all tubes.

(10) Vortex THOROUGHLY.

(11) Centrifuge all assay tubes at 2300-2500 rpm (1000 g) for 15 minutes. Aspirate or decant the supernatant. (If decanting, blot the rim of the test tubes on absorbent paper before turning right side up).

(12) Count the precipitate in a gamma counter.

IX. PROTOCOL

Reagent addition sequence

Tube No.	Description	(1) Steroid Diluent (mL)	(2) Calibrator or Diluted Unknown (mL)	(3) CpB* ^{125}I (mL)	(4) Anti-CpB* (mL)	VORTEX AND INCUBATE FOR 2 HOURS AT ROOM TEMP	(5) Precipitating Solution (mL)	CENTRIFUGE, ASPIRATE OR DECANT / BLOT, AND COUNT
1	NSB	0.3	0	0.2	0		0.5	
2	NSB	0.3	0	0.2	0			
3	0	0.1	0		0.2			
4	0	0.1	0		0.2			
5	25 ng/mL	0	0.1					
6	25 ng/mL							
7	50 ng/mL							
8	50 ng/mL							
9	100 ng/mL							
10	100 ng/mL							
11	250 ng/mL							
12	250 ng/mL							
13	500 ng/mL							
14	500 ng/mL							
15	1000 ng/mL							
16	1000 ng/mL							
17	Control I							
18	Control I							
19	Control II							
20	Control II							
21	Unknown Serum							
22	Unknown Serum	▼	▼	▼	▼		▼	

* CORTICOSTERONE

X. CALCULATIONS

A. Take the average of all duplicate tubes. Subtract the averaged NSB (blank) counts from the averages obtained. This yields the corrected values. Divide the corrected values by the corrected zero calibrator value to obtain the percent bound.

B. Formula

$$\%B/Bo = \frac{\overline{CPM}\text{ (sample)} - \overline{CPM}\text{ (NSB)}}{\overline{CPM}\text{ (0 calibrator)} - \overline{CPM}\text{ (NSB)}} \times 100$$

$\overline{CPM}$ = Average counts of duplicates

Sample = Particular serum or calibrator being calculated

NSB = Non-specific binding tube (also known as blank tube)

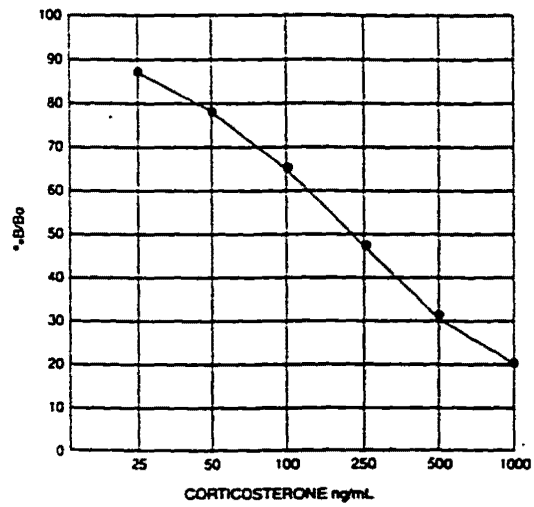
0 Calibrator = 0 tube (also known as the 100% binding tube)

C. Plot percent bound versus the concentration of corticosterone for all the calibrators (25-1000 ng/mL). This yields the calibrator curve. Sample values may then be read directly from this curve.

XI. CALIBRATOR CURVE

NOTE

This curve serves only as an example. Corticosterone values should not be derived from it.



XII. SAMPLE ASSAY

SAMPLE	CPM	AVG. CPM	AVG.-NSB CPM	%B/Bo	RESULT (ng/mL)
NSB (blank)	1085 917	1001			
0 ng/mL	21411 21083	21247	20246	100	
25 ng/mL	19165 18601	18883	17882	88	
50 ng/mL	17142 17026	17084	16083	79	
100 ng/mL	14392 13971	14181	13180	65	
250 ng/mL	10335 10542	10438	9437	47	
500 ng/mL	7363 7366	7364	6363	31	
1000 ng/mL	4880 5046	4963	3962	20	
Control I	9286 9261	9273	8272	41	320
Control II	12356 12228	12292	11291	56	160
Control III	17598 18007	17802	16801	83	38

XIII. SAMPLE CALCULATIONS

$$\text{Control I} = \frac{\overline{\text{CPM (sample)}} - \overline{\text{CPM (NSB)}}}{\overline{\text{CPM (0 calibrator)}} - \overline{\text{CPM (NSB)}}} \times 100$$

$$= \frac{9273 - 1001}{21247 - 1001} \times 100$$

$$= \frac{8272}{20246} \times 100$$

$$= 41\%$$

XIV. EXPECTED NORMAL CONCENTRATIONS

Corticosterone concentrations in mice and rats observed by this RIA are similar to those observed by other traditional but more cumbersome methods such as HPLC, gas chromatography combined with mass spectrometry, fluorometric methods, and other RIA methods. [1,2,5] Because concentrations in mice and rats can vary greatly according to handling techniques and sample collection methods, it is best for each laboratory using this method to determine its own ranges of normal values for baseline and simulated samples. Generally a baseline range of 50 to 300 ng/mL and 50 to 400 ng/mL can be expected for mice and rats respectively depending on the time of day the sample is taken. [1]

XV. PERFORMANCE CHARACTERISTICS

A. RECOVERY OF EXOGENOUS CORTICOSTERONE

To demonstrate the accuracy of the ImmuChem™ Corticosterone-^{125}I method, known amounts of corticosterone were added to aliquots of Steroid Diluent and serum samples previously assayed by a ^{3}H method. These values were then confirmed by the Corticosterone-^{125}I method described herein. The following data were obtained:

Sample	Actual CpB conc. (ng/mL)	Amount of CpB added (ng/mL)	Amount of CpB expected (ng/mL)	Amount of CpB recovered (ng/mL)		% CpB recovered
Steroid Diluent	0.0	50	50	53		106.0
		100	100	95		95.0
		200	200	190		95.0
		400	400	390		97.5
					Average	98.4
B-12	10	50	60	64		106.7
		100	110	95		86.4
		200	210	210		100.0
		400	410	410		100.0
					Average	98.3
PA	147	50	197	220		111.7
		100	247	260		105.3
		200	347	350		100.9
		400	547	600		109.7
					Average	106.9
R400	342	50	392	370		94.4
		100	442	430		97.3
		200	542	540		99.6
		400	742	710		95.7
					Average	96.7
				Overall	Average	100.1

B. PARALLELISM

To demonstrate assay parallelism, five (5) rat serum samples were diluted with Steroid Diluent and assayed. The following data were obtained:

Sample	Undiluted ng/mL	1:2 ng/mL	1:4 ng/mL	1:8 ng/mL
R400	330	180x2=360	81x4=324	43x8=344
DG-23	350	185x2=370	93x4=372	50x8=400
DG-21	450	230x2=460	115x4=460	55x8=440
DG-13	480	230x2=460	120x4=480	62x8=496
DG-22	1100	580x2=1160	280x4=1120	150x8=1200

C. REPRODUCIBILITY

The precision of the method was determined by evaluating replicates of in-house rat serum pools. The following data were obtained:

(1) INTRA-assay variation (n=10)

	R4	PA	DG-4
	360	152	48
	380	168	52
	360	146	48
	360	152	40
	380	174	44
	380	174	40
	400	174	44
	350	168	48
	360	174	40
	350	180	52
Mean	370	166	45.6
S.D.	16.3	11.8	4.7
C.V.	4.4%	7.1%	10.3%

(2) INTER-assay Variation (n=15)

	PA	DG-6	DG-13
	166	128	480
	160	112	480
	156	116	460
	152	115	500
	143	112	520
	146	124	435
	161	130	460
	150	135	470
	160	115	500
	180	122	465
	150	105	490
	145	125	510
	170	122	430
	167	110	410
	180	110	420
Mean	158	119	469
S.D.	10.3	8.6	33.4
C.V.	6.5%	7.2%	7.1%

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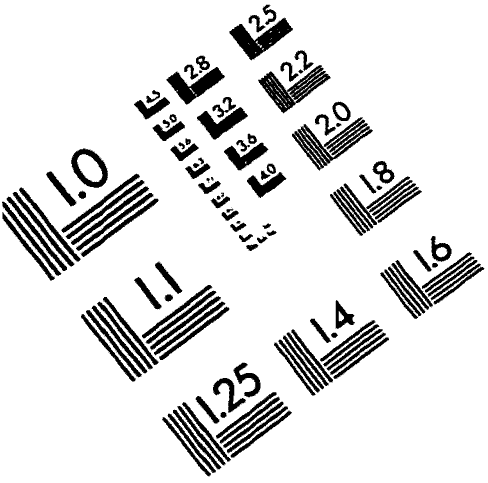
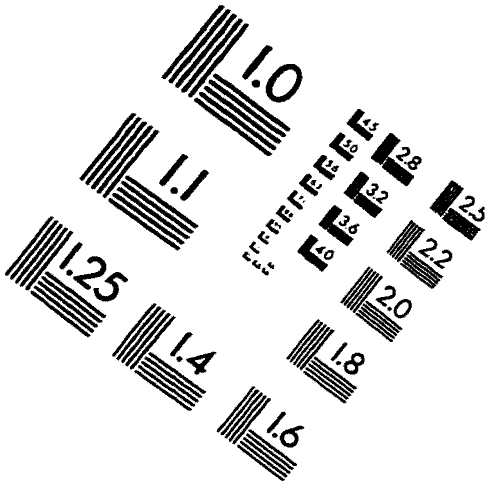
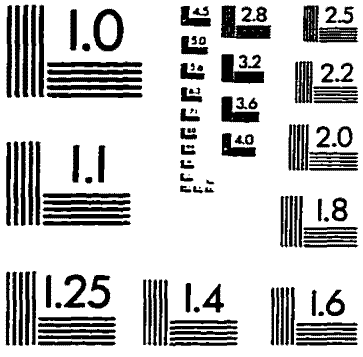
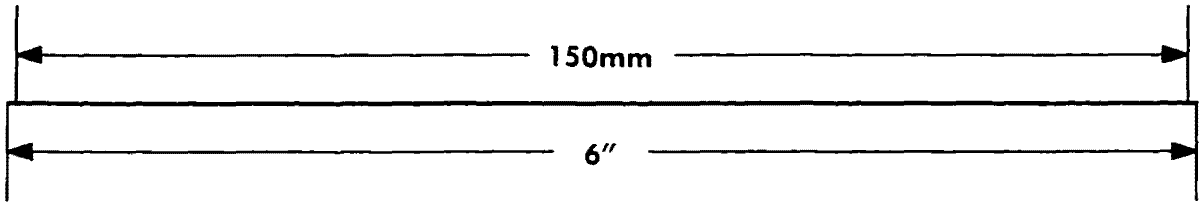
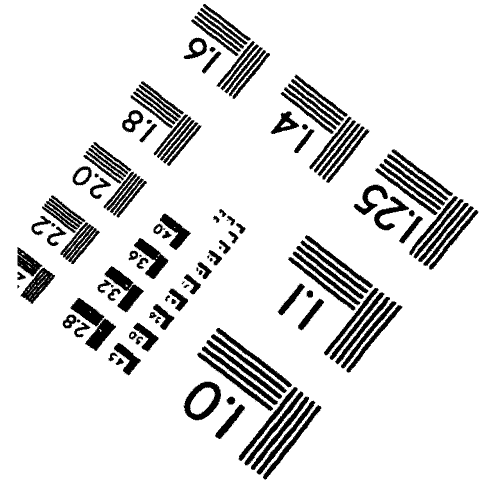
IMAGE EVALUATION TEST TARGET (QA-3)

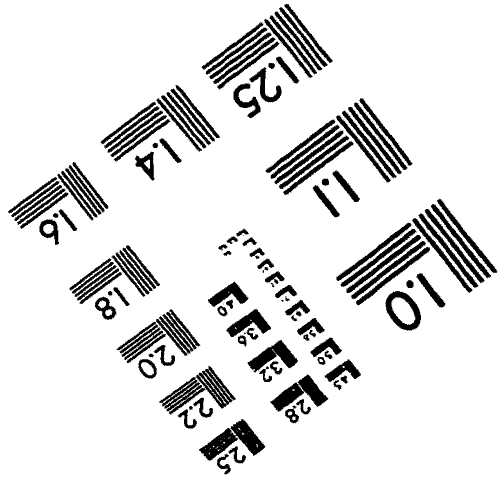












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